Visfatin and Fetuin-A: Novel Markers for Endothelial Dysfunction in Chronic Kidney Disease

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Abstract: Background: Endothelial dysfunction (ED) has a major role in the cardiovascular outcome of patients with chronic kidney disease (CKD). The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation. Visfatin is an adipocytokine that has recently generated much interest and could contribute to endothelial dysfunction. Fetuin-A may be one of the contributing factors for the development of ED in CKD patients. In addition, fetuin-A 256Ser/ Ser (allele G) might affect serum fetuin- A levels. The aim of the present work is to study the role of visfatin and fetuin-A in patients with different stages of CKD in correlation with the level of NO (nitrite /nitrate) as a settled marker of endothelial dysfunction. Also, to study the relation between fetuin-A gene polymorphisms and the susceptibility to ED in patients with CKD in different stages; and identifying the effect of fetuin-A gene polymorphisms on the level of serum fetuin-A in CKD patients. **Methods**: the present study included sixty patients at different stages of CKD with age range from 18 to 60 years old. All patients were non-diabetic and arranged in five groups according to the stage of CKD assessed GFR from stage 1 to 5 representing the groups from I to V. Serum visfatin and serum fetuin-A were measured using ELISA technique and serum NO was measured as (total nitrate and nitrite) using the Griess reaction. Serum levels of glucose, triglycerides, total cholesterol and HDL-cholesterol were estimated by enzymatic colorimetric methods. LDL-cholesterol was then calculated using Friedewald's formula. Genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using polymerase chain reaction (PCR) technique was performed. **Results**: A statistically significant elevation of serum total nitrate and nitrite and serum visfatin in CKD patients compared to controls respectively, while serum fetuin-A showed statistically significant decrease in CKD patients compared to the control group. Serum total nitrate and nitrite levels were significantly increased in all stages of CKD, while serum visfatin was significantly increased in stages 2 to 5 of CKD. Serum fetuin-A showed significant decrease in stages 2 to 5 of CKD. There was no statistically significant difference between the studied CKD cases and the control group as regards to the frequencies of the three genotypes of fetuin–A (C → G); Thr256Ser polymorphism. In both CKD patients and the control group, the distribution of the fetuin-A (C → G); Thr256Ser gene polymorphisms did not show significant correlation with low serum fetuin-A levels. A significant positive correlation was found between serum levels of total nitrate and nitrite and serum levels of (visfatin, triglycerides, total cholesterol, LDL-cholesterol), while A significant negative correlation was found between serum levels of total nitrate and nitrite and serum levels of (fetuin-A and HDL-cholesterol) in CKD patients. Stepwise regression analysis revealed that the strongest predictors of endothelial dysfunction were found to be serum visfatin and HDL-cholesterol as they could explain significantly 52% of the changes in total nitrate and nitrite. **Conclusion:** The results of the present study suggest that high serum levels of visfatin and total nitrate and nitrite in CKD patients may contribute to impaired endothelial functions in CKD. Visfatin and fetuin-A may be novel markers for endothelial dysfunction in CKD patients and in diagnosis of early stages of CKD and they may play a role in uremia-related atherosclerosis. The distribution of the fetuin-A (C → G); Thr256Ser gene polymorphisms may does not affect serum fetuin-A levels.

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1. Introduction

In renal failure, endothelial dysfunction (ED) and atherosclerosis are almost universal, as well as cardiovascular complications. Cardiovascular diseases (CVD) remain the major cause of morbidity and mortality in chronic kidney disease (CKD) patients[(1)] . Traditional cardiovascular risk factors including dyslipidemia, hypertension, smoking and diabetes mellitus are highly prevalent in these patients [(2)]. Nontraditional biomarkers, for example, markers of inflammation, ED, myocardial necrosis and left ventricular remodeling , have been associated with increased cardiovascular event rates and mortality risk in population with and without CKD[(3)].

Endothelial dysfunction (ED) represents an obligatory, prodromal phase in the atherosclerotic process. Endothelial dysfunction may be also responsible for accelerated atherosclerosis in patients with CKD. Endothelial dysfunction is characterized by shift of the action of the endothelium towards reduced vasodilatation and a proinflammatory state denoting impairment of endothelium-dependent vasodilatation[4] . The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation[5] . The endothelium-derived nitric oxide (NO) is synthesized from the substrate L-arginine via endothelial NO synthase (eNOS) and plays a crucial role in regulating a wide spectrum of functions in the cardiovascular system, including vasorelaxation, inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, as well as platelet aggregation. Physical or biochemical injury to the endothelium impairs production and/or function of endothelium-derived vasoprotective mediators of vascular health, such as NO, resulting in increased vascular contraction, enhanced thrombus formation and exacerbated SMC proliferation and migration. It is, therefore, not surprising that loss of endothelial NO function is associated with several cardiovascular disorders, including atherosclerosis [6,7] .

The adipose tissue is a complex organ with function far beyond the mere storage of energy. Indeed, it secretes a number of adipokines. Visfatin is one of the adipokines (also known as adipocytokines) which is a family of secreted proteins released by fat cells that regulate a variety of physiological and pathological processes[8,9] . Visfatin was found to be up-regulated in visceral fat in parallel with insulin resistance[10,11].Visfatin is also called nicotinamide phosphor-ribosyltransferase (Nampt) an enzyme that catalyzes the first step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide. Visfatin which is protein in nature has also been reported to be a pre-B cell colony-enhancing factor (PBEF) that promotes B cell maturation and inhibits neutrophil apoptosis [12,13] . Therefore, visfatin/PBEF/Nampt appears to be a multifunctional protein acting as a hormone, cytokine and/or enzyme [14, 15]. Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme that catalyzes the first step in the biosynthesis of NAD from nicotinamide. This protein was originally cloned as a putative pre-B cell colony-enhancing factor (PBEF) and also found to be a **vis**ceral **fat**-derived adipok**in**e (**visfatin**). [16]. As a multifunctional protein, visfatin plays an important role in immunity, metabolism, aging, inflammation and responses to stress [17] . However, the pathophysiological role of visfatin is not completely understood .Visfatin is implicated in the transcriptional regulatory activity of a variety of cellular processes, including stress, cytokine responses, differentiation and metabolism [18,19] . One function of visfatin is the regulation of inflammatory and immunomodulating processes. Visfatin can induce the cellular expression of inflammatory cytokines, such as (interleukin-1β) IL-1β, Tumour necrosis factor-α (TNF-α) and (interleukin-6) IL-6 [20] . Deteriorating renal function may increase overall inflammatory responses because of the decreased renal clearance of factors that are directly or indirectly involved in inflammation. Inflammation has also been implicated in vascular diseases as acute myocardial infarction and thrombotic stroke. Chronic renal disease is a highly associated with atherogenic disease and subclinical systemic inflammation [21] . Visfatin may play an important role in atherogenesis and plaque destabilization and due to the fact that endothelial cell damage or injury is invariably associated with clinical conditions such as thrombosis, hypertension, renal failure and atherosclerosis, visfatin may be considered as a novel modulator for endothelial dysfunction [22] .

Fetuin-A is an acidic glycoprotein synthesized by the liver, it is expressed also in osteoblasts, the tongue and the placenta [23,24].Fetuin-A is part of the cystatin superfamily of cysteine protease inhibitors [25] The fetuin-A gene is located at chromosome 3q27 [26]

Fetuin-A, plays an important role in the development of specific organs (bone marrow, brain, gonads and liver).[27]Fetuin-A is a negative acute phase protein.[28] Fetuin-A has role as a protease inhibitor, and acts as an inhibitor of matrix metalloproteinases. [29] Fetuin-A down regulates the synthesis of proinflammatory cytokines and prevents excessive inflammation [30] . Fetuin-A acts as circulating inhibitor of calcification. [31]

In renal failure, hyperphosphataemia was recognized as an independent risk factor for cardiovascular disease[32] . It was believed that the mineral homoeostasis which is disturbed in CKD, results in an elevated serum calcium phosphate (Ca-Pi) product sustaining calcium phosphate precipitation [33] .

Fetuin-A accumulates in the skeleton due to a high affinity to hydroxyapatite. Fetuin-A binds to bone morphogenic protein-2 and transforming growth factor β inhibiting mineralization and suppressing the expression of bone matrix proteins. It inhibits the de novo formation and precipitation of the apatite precursor mineral, basic calcium phosphate (BCP) [34] Fetuin-A can inhibit undesirable calcification in circulation without inhibiting bone mineralization [35] It was hypothesized that fetuin-A on the surface of these mineral colloids has two functions (i) it reduces diffusion of ions to the mineral core and (ii) it prevents particle aggregation [36] . So that fetuin-A may act as a systemic inhibitor of ectopic calcification.

Defective endothelial function, an initial step in the development of atherosclerotic plaque, is prevalent in moderate to advanced CKD [37] . These data in CKD patients indicate that fetuin-A may be one of the contributing factors for the development of endothelial dysfunction in CKD patients[38] , specific polymorphisms of fetuin-A gene were demonstrated to influence circulating levels of fetuin-A [39] .

The aim of the study was to assess the role of serum visfatin and fetuin-A as novel markers of ED in patients with different stages of CKD, in correlation with the marker of endothelial damage NO (total nitrates and nitrites); as well as studying the relation between fetuin-A gene polymorphisms and the susceptibility to ED in patients with CKD in different stages and the effect of fetuin-A gene polymorphisms on the level of serum fetuin-A.

2. Materials and Methods

The present study included sixty patients at different stages of CKD defined according to the National Kidney Foundation (NKF) – Kidney Disease Outcomes Quality Initiative (KDOQI) classification[40] with age range from 18 to 60 years old. All patients were non-diabetic and arranged in five groups according to the stage of CKD assessed by glomerular filtration rate (GFR) as follows:

Group I: stage 1(GFR≥90 ml/min/1.73m^2).
Group II: stage 2 (GFR from 60 to 89 ml/min/1.73m^2).
Group III: stage 3 (GFR from 30 to 59 ml/min/1.73m^2)
Group IV: stage 4 (GFR from 15 to 29 ml/min/1.73m^2).
Group V: stage 5 (GFR <15 ml/min/1.73m^2).

Each group included twelve patients. Also, **Group VI** twelve matched healthy volunteers with matched age and sex were included to serve as healthy controls.

The patients were chosen from the main University Hospital of Alexandria. In order to minimize any confounding effects of conditions that may influence ED, patients with overt congestive heart failure, valvular heart disease, acute coronary syndrome, atrial fibrillation, smokers, nephrotic syndrome, patients taking angiotensin converting enzyme inhibitors, angiotensin receptor blockers, statins or supplemental vitamin pills were excluded. In addition, patients with a prior diagnosis of diabetes or with a fasting glucose level greater than 126 mg/dl were also excluded. The subjects were told to avoid foods high in nitrate (i.e. spinach, beets, cabbage, cauliflower and lettuce) for 3 days before taking blood samples and were asked to fast overnight before sampling blood [41] .

The study was conducted in accordance with the Local Ethics Committee of the Faculty of Medicine, University of Alexandria; an informed consent was obtained from all patients included in the study.

All patients included in this study were generally evaluated depending on the following:

I- Clinical Evaluation: Includes proper history taking and clinical examination particularly stressing on vital sign measurement (blood pressure, pulse and temperature).

II- Calculation of GFR from serum creatinine levels using Cockcraft and Gault (C&G) formula [42]

Glomerular filtration rate (GFR) was determined for proper diagnosis, selection and grouping of cases and controls.

Cockcroft-Gault GFR = (140-age) X (Wt in kg) X (0.85 if female) / (72 X Cr).

Wt=weight, Cr= serum creatinine in mg/dL.

III- Laboratory Investigations

A) Sample collection, storage and preparation:

Five milliliter venous blood were withdrawn from every patient and control in the morning after over night fasting. 500 µl whole blood was separated in an eppendorf containing ethylenediamine tetraacetic acid (EDTA) 5% and stored at –20°C for genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using Polymerase chain reaction (PCR) technique.

The remaining sample was transferred into disposable plastic tube and was allowed to clot, then was centrifuged at 1200 rpm for 10-15 minutes to separate the serum. The serum was then divided into four aliquots which were kept frozen at -20°C until use. The following laboratory investigations were carried out:

B) Routine Laboratory Investigations:

They included estimation of serum glucose [43], creatinine [44] , triglycerides [45] , total cholesterol [45] and high-density lipoprotein (HDL) cholesterol[46] using enzymatic colorimetric methods. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald's formula. [45]

C) Specific laboratory tests:

1. Determination of serum visfatin [47] using enzyme linked immunosorbant assay (ELISA) (RayBio® Human Visfatin Enzyme Immunoassay, US) and serum fetuin-A[48] also using ELISA (BioVendor Human Fetuin-A ELISA,Germany).
2. DNA extraction and genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using PCR technique [49] .
3. Estimation of NO (nitrate and nitrite) based on the Griess reaction [50,51] .

DNA extraction from blood, amplification of fetuin -A gene by PCR and (Thr256Ser) mutation analysis by restriction endonuclease enzyme sacI[49]

DNA extraction:

DNA was purified from whole blood using Axygen Prep Blood Genomic DNA Miniprep Kit for the purification of genomic DNA from whole blood. This method was based on the efficient release of genomic DNA from anti-coagulated whole blood by a special cell lysis and heme/protein precipitation buffer coupled with the selective adsorption of the genomic DNA to a special AxyPrep column. The purified genomic DNA was eluted in a low-salt Tris buffer containing 0.5 mM EDTA which enhanced DNA solubility and helped to protect the high molecular weight DNA against subsequent nuclease degradation. The eluted genomic DNA was subjected to PCR amplification of the fetuin-A gene.

PCR amplification

DreamTaq™ Green PCR Master Mix (fermentas life sciences)

DreamTaq™ Green PCR Master Mix (2X) was a ready to use solution containing DreamTaq™ DNA polymerase, optimized DreamTaq™ Green buffer, MgCl2 and dNTPs. The master mix was supplemented with two tracking dyes and a density reagent that allow for direct loading of the PCR product on a gel. The dyes in the master mix did not interfere with PCR performance and were compatible with downstream applications such as restriction digestion.

For a total 25µl reaction volume:

DreamTaq™ Green PCR Master Mix (2X) 12.5 µl.
Forward Primer 1µl.
Reverse Primer 1 µl.
Extracted DNA 5 µl .
Water nuclease-free 5.5 µl.

Primers:

A pair of primers was utilized to amplify the fetuin-A gene. The lyophilized primers were purchased from Fermentas Life Sciences. The lyophilized primers were reconstituted by addition of sterile water to a final concentration of 50 picomoles/µl and distributed in aliquots and stored at –20°C.

Primer sense sequence:

F5-GTCACCCCTCCTTGTAAC-3
[Tm (thermodynamic) = 45.6°C]

Reverse primer antisense:

R5-CCCCAATGAGACCACA-3.[Tm = 46°C]

Protocol of amplification:

Tubes were transferred to the thermal cycler (Biometra) where the PCR conditions were as follows:

- Initial denaturation at 95°C for 5 minutes.
- 35 cycles of denaturation at 94°C for 1minute, annealing at 59°C for 1 minute, extension at 72°C for 1 minute.
- Final extension at 72°C for 15 minutes.

Mutation analysis of fetuin-A Thr256Ser (c.766C >G) polymorphism [49]

For mutational analysis of fetuin-A Thr256Ser (c.766C > G); FastDigest® SacI restriction enzyme 100 µl (for 100 reactions) was supplied with 1 ml of 10X FastDigest® Buffer. They were supplied from (Fermentas Life Sciences) and stored at -20°**C.**

Protocol for Fast Digestion of DNA:

Total volume: 30 µl;

- Water nuclease-free 17 µl.
- 10X FastDigest® buffer 2 µl.
- PCR product 10 µl.
- FastDigest® enzyme 1 µl.

They were mixed gently and incubated at 37°C for 60 min.

The digested products were separated on 1.5% agarose gel. Allele C does not contain the *Sac*I site remain undigested as 405 bp fragments, whereas allele G yields 193- and 212-bp fragments.

For Detection of the digested products PCR marker (Fermentas Life Sciences), the DNA fragments range from 50bp to1000 base pairs was used and Gel electrophoresis was performed:

- 12µl of the digested product was slowly loaded into the slots of the submerged gel using a micropipette.
- 8 µl of the TE buffer +2 µl of the50bp ladder+2 µl of loading dye 6X (Amresco) was slowly loaded into one of the slots the submerged gel using a micropipette.
- A 302 nm ultraviolet transilluminator was used for visualization of the DNA bands. Allele C does not contain the *Sac*I site remain undigested as 405 bp fragments whereas allele G yields 193- and 212-bp fragments and they were photographed.

Determination of serum nitric oxide metabolites (nitrite and nitrate) concentrations

I- Nitrate assay [50]: Serum nitrate was quantitated colorimetrically through nitrate reduction by NADPH-dependent nitrate reductase. Serum samples were incubated with FAD (0.2 mmol/L), NADPH (12 mmol/L) and nitrate reductase from Aspergillus species (500 U/L). The reaction was allowed to develop in the dark because of photosensitivity of FAD. Then, the absorbance of samples was recorded at wave length 340 nm.

At 25° C, 100 µl of serum sample were mixed with 250 µl of 100 mmol/L potassium phosphate buffer (PH 7.5), 50 µl of distilled water, 50 µl of 0.2 mmol/L FAD and 10µl of 12 mmol/ L NADPH. 40 µl of 500 U/L nitrate reductase were added and immediately mixed. The reaction was allowed to develop in darkness. After 45 minutes incubation, the absorbance was recorded at 340 nm.

Calculation:

Nitrate concentration in the sample was calculated form the following equation:

Nitrate concentration = Δ Absorbance × factor

Where:

Δ Absorbance = ASB- AS- ARB

ASB =Absorbance of sample blank.

AS =Absorbance of sample

ARB =Absorbance of reagent blank

Factor = VT/VS × 1/L × 1/ε 340 nm × 10^3 = 0.833

VT = total volume of reaction mixture (500 µls)

VS = sample volume

L = light path [1cm, ε 340 nm = millimolar absorpitivity of NADPH at 340 nm (6.22 L. $mmol^{-1}$ cm^{-1})]

10^3 = conversion from mmol/L to µmol/L

II- Nitrite assay [51]: Serum nitrite was quantitated colorimetrically after reaction with Griess reagents. Griess assay reagents (1% sulfanilamide, 0.5% naphthylethylenediaminedichloride and 2.5% phosphoric acid) were first mixed and incubated with samples or standard to form a purple azo dye. Then the absorbance was measured at wave length 543 nm. Nitrite concentration was determined from a linear standard curve.

0.4 ml of each sample was mixed with 0.8 ml of freshly prepared 1% sulfanilamide in 2.5% phosphoric acid and 0.8 ml of 0.5% naphthylethylenediaminedichloride in 2.5% phosphoric acid. The absorbance was measured at 543 nm after incubation for 10 minutes at room temperature in dim light. Concentration was determined from a linear standard curve between 2.5 and 30µM sodium nitrite. NO was estimated as the sum of total nitrates and nitrites.

Statistical analysis

The study data were statistically analyzed using the Statistical Package for Social version program (SPSS program-version 10.0 – SPSS Inc., Chicago, IL. USA). The data were expressed as median, mean ± standard deviation (SD) or proportions (%). Between-groups comparisons were assessed for nominal variables with the χ2-square test while for quantitative variables differences among the groups were analyzed by Mann-Whitney test and Kruskal–Wallis test. Correlations between variables were analyzed by using Pearson's correlation coefficient. Statistical significance was assessed at $P < 0.05$. All calculated P values were two-tailed.

3. Results

The Distribution of patients according to the etiology of CKD in the present study is shown in Table I: 24 patients (40%) were due to hypertension,18 patients (30%) had glomerulonephritis, 10 patients (16.7%) had reflux nephropathy , 2 patients (3.3%) had autosomal dominant polycystic kidney and in 6 patients(10%) the etiology of CKD was unknown.

Table I. Distribution of patients according to the etiology of CKD

The etiology of CKD	Frequency	Percent (%)
Hypertension	24	40
Glomerulonephritis	18	30
Autosomal dominant polycystic kidney	2	3.33
Reflux nephropathy	10	16.67
Unknown	6	10
Total	60	100

In the present study, the age range in the patient group was from 20 to 59years with a mean value of 40.25 ± 10.8 years, while the range in the control group was from 27to 55 years with a mean value of 42.17± 9.321years. Statistical comparison of the subjects' age, showed no significant difference between the two studied groups (Z = 0.932, *p*= 0.338). CKD patients included 31 males (51.7%) and 29 females (48.3%) while the control group included 5 males (41.7%) and7 females (58.3%). Statistical comparison of the subjects' sex, showed no significant difference between the two studied groups (χ^2 =0.4, *p* =0.527). Serum levels of glucose, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol are shown in Table II. Statistical comparisons between CKD patients and the control groups as regards serum levels of glucose, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol were not significant at $p > 0.05$.

In CKD patients, serum total nitrate and nitrite concentration ranged between 32.4 -132.3 µmol/l with a mean value of 76.57±20.38µmol/l (median value of 71.35µmol/l). In the control group, serum total nitrate and nitrite concentration ranged between 31.2 - 53.7µmol/l with a mean value of 42.77±8µmol/l (median value of 41.75µmol/l). There was statistically significant elevation of serum total nitrate and nitrite concentration in CKD patients compared to the control group (Z=-5.101, $p < 0.001$). (Table II)

As regards serum visfatin concentration in CKD patients, it ranged between 21-80ng/ml with a mean value of 46.34±15.52 ng/ml (median value of 43

ng/ml). In the control group, serum visfatin concentration ranged between 21-42 ng/ml with a mean value of 31.62±7.35 ng/ml (median value of 31.7ng/ml). There was statistically significant elevation of serum visfatin concentration in CKD patients compared to the control group (Z=-3.224, *p* =0.001). (Table II).

Serum fetuin-A concentration ranged between 0.01-0.47 g/l with a mean value of 0.23±0.09 (median value of 0.21g/ml) in CKD patients. In the control group, serum fetuin-A concentration ranged between 0.24-0.45 g/l with a mean value of 0.33±0.05 (median value of 0.32g/l). There was statistically significant decrease of serum fetuin-A concentration in CKD patients compared to the control group (Z=-3.325, *p* =0.001). (Table II).

As regards statistical comparison in between CKD groups, in Group V there was statistically significant elevation of serum total nitrate and nitrite levels and serum visfatin levels in comparison with (Group I,II and III); $p < 0.05$. Also, in Group IV there was statistically significant elevation of serum total nitrate and nitrite levels in comparison with Group I and significant elevation of serum visfatin in comparison with Group I and Group II; $p \leq 0.05$(Table III.)

In Group III there was statistically significant elevation of serum visfatin levels in comparison with Group I ; $p \leq 0.05$,but no statistically significant difference was found between Group III or II; $p > 0.05$. (Table III.)

As regard serum fetuin-A levels there was statistically significant decrease in Groups III, IV and V in comparison with Group I; $p < 0.05$. (Table III.)

Table II: Statistical comparisons between CKD patients and the control group as regards routine laboratory data, serum levels of total nitrate and nitrite, visfatin and fetuin-A.

Parameters	CKD patients (n=60)	Control (n=12)	Statistical test	*P* value
Serum glucose(mg/dl) Mean ± SD	83.57±6.63	80.25±5.53	Z=-1.707	0.088 NS
Median	81	85		
Range	70- 99	70-88		
Serum triglycerides(mg/dl) Mean ± SD	161.43±66.96	130.85±59.56	Z=-1.463	0.143 NS
median	164.9	150		
range	68-276	71.3-250		
Serum total cholesterol(mg/dl) Mean ± SD	173.4±46.01	150.5±24.3	Z=-1.709	0.087 NS
Median	173.44	150		
Range	99-291	99.2-189.9		
Serum LDL-cholesterol (mg/dl) Mean ± SD	114.13±45.39	88.37±35.89	Z=-1.712	0.087 NS
Median	107.6	91.42		
Range	34.44-191.5	34.44-165		
Serum HDL-cholesterol(mg/dl) Mean ± SD	43.48±45.39	50.2±10.45	Z=-1.501	0.133 NS
Median	42.6	54.1		
Range	12-67	32.5-67		
Serum total nitrate and nitrite (µmol/l) Mean ± SD	76.57±20.38	42.77±8	Z=-5.101	<0.001**
Median	71.35	41.75		
Range	32.4 -132.3	31.2-53.7		
Serum visfatin (ng/ml) Mean ± SD	46.34±15.52	31.62±7.35	Z=-3.224	0.001**
Median	43	31.7		
Range	21-80	21-42		
Serum fetuin-A (g/l) Mean ± SD	0.23±0.09	0.33±0.05	Z=-3.325	0.001**
Median	0.21	0.32		
Range	0.01-0.47	0.24-0.45		

Z: for Mann-Whitney test. SD: Standard deviation. NS:Nonsignifiant. **: Highly significant ($p \leq 0.01$).

Table III: Statistical comparison between different groups of CKD as regards serum total nitrate and nitrite (µmol/l), serum visfatin(ng/ml) and serum fetuin-A(g/l)

Parameters	Groups						χ^2 *p* value
	Group I Stage1 (n=12)	**Group II Stage2 (n=12)**	**Group III Stage3 (n=12)**	**Group IV Stage4 (n=12)**	**Group V Stage5 (n=12)**	**Group VI Control (n=12)**	
Serum nitrate & nitrite(µmol/l) Range Mean± SD Median	32.4-122 66.08± 23.65 62.2	51 -96 71.5±15.30 65.75	50.3-96.1 73.56±15.24 69	58.40-100.3 80.9±15.75 82.45	64-132.3 92.89±21.54 90.54	31.20-53.7 42.77±8 41.75	χ^2=35.48 p<0.001**
Z_1 (p)		-0.925 (0.378) NS	-0.925 (0.378) NS	-1.993 (0.045) *	-2.714 (0.006) **	-3.205 (0.001) **	
Z_2 (p)			-0.290 (0.799) NS	-1.328 (0.198) NS	-2.483 (0.012) *	-3.984 (<0.001) **	
Z_3 (p)				-1.328 (0.198) NS	-2.483 (0.012) *	-3.984 (<0.001) **	
Z_4 p)					-1.213 (0.242) NS	-4.158 (<0.001) **	
Z_5 (p)						-4.157 (<0.001) **	
Serum Visfatin(ng/ml) Range Mean± SD Median	21-46 35.08±8.87 39	27-48 38.75±6.9 40	30-61 44.42±9.3 40	23-80 55.82±20.32 60.45	38-80 57.65±14.92 60.50	21-42 31.63±7.35 31.70	χ^2=25.52 p=0.001**
Z_1 (p)		-1.131 (0.266) NS	-2.009 (0.045) *	-2.545 (0.011) *	-3.124 (0.001) **	-1.069 (0.291) NS	
Z_2 (p)			-1.253 (0.219) NS	-2.197 (0.028) *	-2.862 (0.003) **	-2.196 (0.028) *	
Z_3 (p)				-1.740 (0.089) NS	-2.117 (0.033) *	-2.692 (0.007) **	
Z_4 (p)					-0.029 (0.977) NS	-2.714 (0.007) **	
Z_5 (p)						-3.637 (<0.001)**	
Fetuin-A (g/l) Range Mean± SD Median	0.13-0.44 0.30±0.08 0.31	0.17-0.47 0.26±0.10 0.21	0.14-0.29 0.22±0.07 0.23	0.15-0.30 0.20±0.05 0.20	0.01-0.35 0.19±0.11 0.20	0.24-0.45 0.33±0.05 0.33	χ^2=21.59 p=0.001**
Z_1 (p)		-0.752 (0.478) NS	-2.428 (0.014) *	-2.656 (0.007) **	-2.369 (0.017) *	-0.895 (0.378) NS	
Z_2 (p)			-1.158 (0.266) NS	-1.677 (0.094) NS	-1.273 (0.219) NS	-2.314 (0.021) *	
Z_3 (p)				-0.145 (0.887) NS	-0.347 (0.755) NS	-3.094 (0.001) **	
Z_4 (p)					-0.058 (0.977) NS	-3.868 (<0.001)**	
Z_5 (p)						-3.235 (0.001) **	

χ2: Chi square for Kruskal Wallis test.
Z_1 : Z for Mann Whitney test between group I and other groups.
Z_2: Z for Mann Whitney test between group II and other groups.
Z_3: Z for Mann Whitney test between group III and other groups.
Z_4: Z for Mann Whitney test between group IV and other groups.
Z_5: Z for Mann Whitney test between group V and other groups.

* : Statistically significant ($p \leq 0.05$).
**: Highly significant ($p \leq 0.01$).
SD: Standard deviation.

Matrix correlations between age, serum levels of total nitrate and nitrite, visfatin, fetuin-A, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol in CKD patients are recorded in Table 4. A significant positive correlation was found between serum levels of total nitrate and nitrite and

serum levels of visfatin, triglycerides, total cholesterol and LDL-cholesterol; (r = 0.475; p <0.001, r = 0.320; p =0.006, r = 0.258; p = 0.029 and r=0.346; p =0.003; respectively), while a significant negative (inverse) correlation was found between serum levels of total nitrate and nitrite and serum levels of fetuin-A and HDL-cholesterol (r = -0.325; p =0.005 and r = -0.283; p =0.016; respectively). A significant positive correlation was found between serum levels of visfatin and serum levels of triglycerides, total cholesterol and LDL-cholesterol (r = 0.453; P<0.001, r = 0.391; p =0.001and r = 0.463; p = <0.001; respectively), while a significant negative (inverse) correlation was found between serum levels of visfatin and fetuin-A (r = -0.269, p =0.022). Also, A significant negative (inverse) correlation was found between serum levels of fetuin-A and serum levels of triglycerides, total cholesterol and LDL-cholesterol (r = -0.310; p =0.008,r = -0.256; p =0.03 and r = -0.420; p = <0.001; respectively), while a significant positive correlation was found between serum levels of fetuin-A and HDL-cholesterol (r = 0.416, p <0.001).(Table 4)

Univariate correlation between serum levels of total nitrate and nitrite and visfatin in CKD patients showed a significant positive correlation between the two parameters in CKD patients (r^2 = 0.225; p <0.001) (Figure 1). While, a univariate correlation between serum fetuin-A and both serum levels of total nitrate and nitrite and serum levels of visfatin with in CKD patients showed a significant negative correlation between the two parameters (r^2 = 0.106, p=0.005; r^2 = 0.091, p =0.01, respectively) (Figures 2,3).

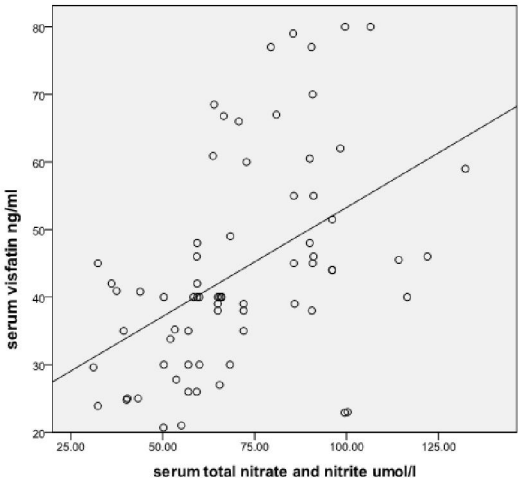

Figure (1). Univariate correlation between serum levels of total nitrate and nitrite and visfatin in CKD patients. r2 = 0.225, p<0.001

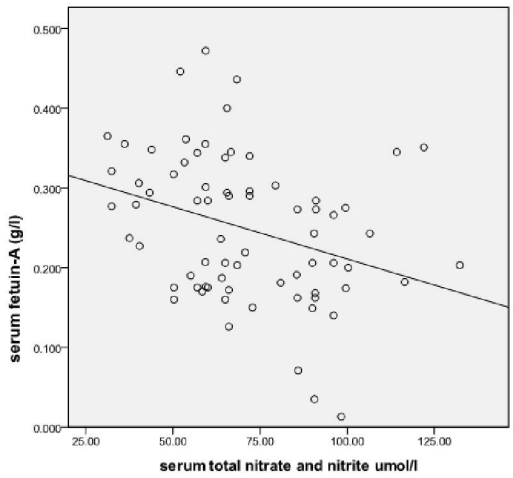

Figure (2). Univariate correlation between serum levels of total nitrate and nitrite and fetuin-A in CKD patients. r2 = 0.106, p=0.005

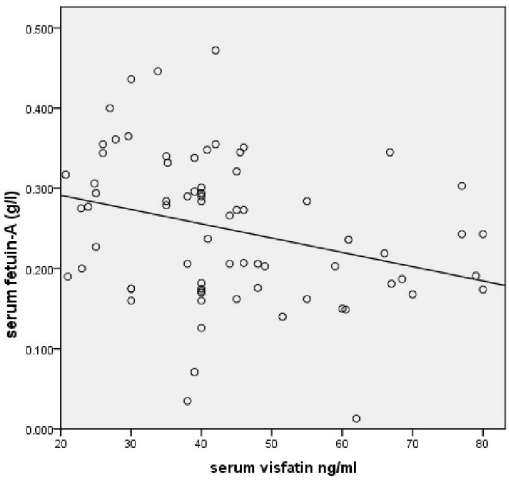

Figure (3). Univariate correlation between serum levels of visfatin and fetuin-A in CKD patients. r2 = 0.091, p=0.01

Table IV. Matrix correlation between age, serum levels of total nitrate and nitrite, visfatin, fetuin-A, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol in CKD patients.

		Total nitrate and nitrite μmol/l	Visfatin ng/ml	Fetuin-A (g/l)	Triglycerids (mg/dl)	Total cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Age	r	0.110	0.188	0.100	0.198	0.185	0.197	-0.009
	p	0.403 NS	0.151 NS	0.447 NS	0.129 NS	0.158 NS	0.132 NS	0.943 NS
Total nitrate and nitrite (umol/l)	r		0.475	-0.325	0.320	0.258	0.346	-0.283
	p		<0.001 **	0.005 **	0.006 **	0.029 *	0.003 **	0.016 *
Visfatin (ng/ml)	r			-0.269	0.453	0.391	0.463	-0.194
	p			0.022 *	<0.001 **	0.001 **	<0.001 **	0.102 NS
Fetuin-A (g/l)	r				-0.310	-0.256	-0.420	0.416
	p				0.008 **	0.03 *	<0.001 **	<0.001 **
Triglycerides (mg/dl)	r					0.626	0.926	-0.378
	p					<0.001 **	<0.001 **	0.001 **
Total cholesterol (mg/dl)	R						0.704	-0.125
	p						<0.001 **	0.294 NS
LDL-cholesterol (mg/dl)	r							-.555
	p							<0.001 **

r: Pearson correlation coefficient. NS: non significant *: Statistically significant ($p \leq 0.05$). **: Highly significant ($p \leq 0.01$).

In order to clarify predictors of endothelial dysfunction in the CKD patients, a stepwise regression model was done. The strongest predictors of endothelial dysfunction measured as serum total nitrate and nitrite were visfatin and HDL-cholesterol (beta= 0.635, p <0.001 and beta=-0.388, p =0.0039; respectively) ,while r^2 of the model was 0.52. This means that 52% of the changes in serum total nitrate and nitrite could be explained and accounted by serum visfatin plus serum HDL-cholesterol.

Serum total nitrate and nitrite can be calculated from the equation:

Y= a + b1x1+ b2x2

Y= serum total nitrate and nitrite umol/l

a=60.339, b1=0.635, x1= serum visfatin ng/ml, b2=-0.388, x2= serum HDL-cholesterol (mg/dl)

Results of gene polymorphism on fetuin –A (C⟶ G); Thr256Ser (Table V, Figure 4)

Restriction enzyme analysis was attempted for detection of fetuin-A gene restriction fragment length polymorphism(C ⟶G); Thr256Ser at *Sac*I recognition site. Allele C does not contain the *Sac*I site and remained undigested as 405 bp fragments, whereas allele G contained the *Sac*I recognition site and yielded 193- and 212-bp fragments. There are three possible genotypes for the fetuin-A: i.e. Thr/Thr**(**Allele C), Thr/Ser (heterozygote) and Ser/Ser (Allele G); (homozygote for absence of the sacI site, heterozygote and homozygote for the presence of the sacI site; respectively) (Figure 4).

The frequencies of the three genotypes in the CKD patients were: Thr/Thr **(**Allele C) 45 patients(75%), Thr/Ser (heterozygote) 12 patients (20%) and Ser/Ser (Allele G) 3 patients(5%), while in the control group, the frequencies were Thr/Thr**(**Allele C) 8 subjects(66.7%), Thr/Ser (heterozygote) 3subjects (25%) and Ser/Ser(Allele G) 1 subject (8.3%). There was no statistically significant difference between CKD patients and the control group according to the frequencies of the three fetuin–A genotype polymorphisms (C ⟶G); Thr256Ser ($\chi 2$=0.414, p =0.813) (Table V).

Table V: Statistical comparison between CKD patients and the control group according to the frequencies of fetuin –A genotype polymorphisms (C ⟶G); Thr256Ser .

	Thr/Thr (Allele C)	**Thr/Ser (Heterozygote)**	**Ser/Ser (Allele G)**	**total**	χ^2	**(*p*)**
cases	45(75.0%)	12(20.0%)	3(5.0%)	60(100%)	0.414	0.813 NS
control	8 (66.7%)	3(25.0%)	1(8.3%0)	12(100%)		
Total	53(73.6%)	15(20.8%)	4(5.6%)	72(100%)		

χ^2 =Pearson Chi-Square. NS: non significant

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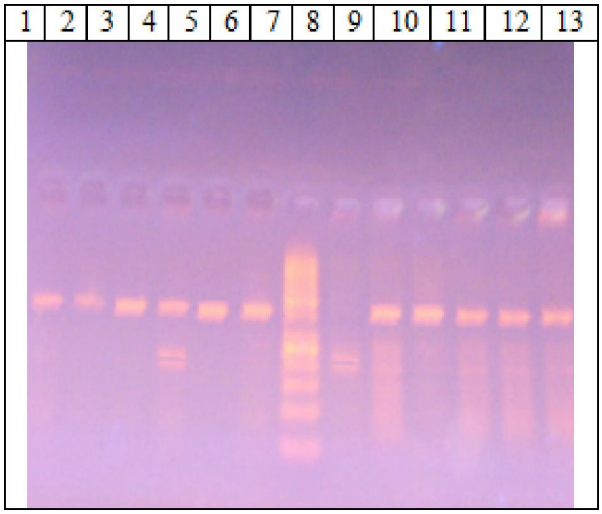

Figure 4: Ethidium bromide stained agarose gel electrophoresis of PCR amplified fetuin-A gene (C ⟶ G); Thr256Ser following sac I restriction digestion for some samples.

Lane 7: DNA size marker 50 – 1000 bp.
Lane 8: Allele G (Ser/Ser) homozygote for the presence of the sacI site which digests the 405 bp fragment into 193 and 212 bp fragments in a homozygote patient.
Lane 1,2,3,5,6,9,10,11,12,13: Allele C (Thr/Thr) undigested as 405 bp. fragments homozygote for absence of the sacI site.
Lane 4: heterozygote patient (Thr/Ser).

Results of the effect of fetuin-A (C ⟶ G); Thr256Ser gene polymorphisms on the level of serum fetuin-A (Table VI).

In both CKD patients and the control group, the distribution of the fetuin-A gene polymorphisms (C ⟶ G); Thr256Ser did not show statistically significant difference between fetuin-A gene polymorphisms and serum fetuin-A levels ($\chi 2$=4.305;P=0.116, $\chi 2$=1.756; p =0.416; respectively).

Showing:

Table (VI). Statistical comparison between the distribution of the fetuin-A (C ⟶ G);Thr256Ser gene polymorphisms and median serum fetuin-A levels in CKD patients and the control group.

Fetuin-A (g\l)	Thr/Thr (Allele C)	Thr/Ser (Heterozygote)	Ser/Ser (Allele G)	$\chi 2$	(p)
CKD cases(n)	45	12	3	4.305	0.116 NS
median(g\l)	0.243	0.179	0.19		
Control(n)	8	3	1	1.756	0.416 NS
median(g\l)	0.324	0.348	0.279		

χ^2: Chi square for Kruskal Wallis test. NS: non significant.

4. Discussion

Endothelial dysfunction (ED) represents an obligatory, prodromal phase in the atherosclerotic process. Endothelial dysfunction may be also responsible for accelerated atherosclerosis in patients with chronic renal failure. Indeed, chronic renal disease is a highly atherogenic disease and state. Thus, the presence of even minor kidney dysfunction has recently been recognized as a significant risk factor for subsequent CVD and death [52] . The etiology of ED is complex and involves dysregulation of multiple pathways. The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation, which may be attributed to decreased production or activity of nitric oxide[53] . To assess the generation of nitric oxide (NO); measurement of NO production in vivo is difficult because of its short half-life. Consequently, its metabolite total serum nitrate and nitrite has been used as a surrogate marker for estimating NO production [54] .

The present study showed significant elevation of total serum nitrate and nitrite in CKD patients compared to control group (Z=-5.101, $p < 0.001$). In all CKD groups serum total nitrate and nitrite levels was statistically significant than the control group ($\chi 2$=35.48, $p < 0.001$). This is in accordance with several studies [55-57] that reflected the elevation of NO in chronic renal failure and hemodialysis. To our knowledge this study is pioneer to demonstrate significant elevation of total serum nitrate and nitrite in early stages of CKD stage I and II (group I and II CKD patients). The underlying molecular mechanisms for the reduced action of NO has been investigated and attributed to a decrease in NO bioavailability which may be caused by decreased expression of eNOS, lack of substrate or cofactors for eNOS, alteration in the signaling pathways activating eNOS, and/or accelerated degradation of NO by reactive oxygen species [58], increased inflammation-induced inducible nitric oxide synthase (iNOS) expression and thus NO.

Recently **Ueda *et al.*** [59] has looked for CKD reported increased asymmetric dimethyl arginine (ADMA) levels in CKD (even in CKD stage I patients) who did not have classical CVD risk factors, suggesting that NO metabolism is impaired in CKD. ADMA, an endogenous inhibitor of NO, its increase is associated with reduced nitric oxide synthesis and vascular dysfunction [59] . In the present study a significant positive correlation was found between serum levels of total nitrate and nitrite and triglycerides, total cholesterol, LDL-cholesterol. (r = 0.320, p =0.006; r = 0.258, p = 0.029; r=0.346; 0.003, respectively), while a significant negative correlation was found between serum levels of total nitrate and nitrite and HDL-cholesterol (r = -0.283, p =0.016, respectively). In agreement with our result **Patil *et al.*** [60] found that altered nitric oxide level may be related to advanced endothelial dysfunction, increased nitric oxide end-products associated with low HDL levels and increased oxidative stress and suggested that elevated levels of NO promote the peroxidation of the lipid moiety and induce immune responses and inflammatory reactions that cause cell damage. Meanwhile HDL cholesterol is considered as an independent, strong inverse predictor of cardiovascular events [61] .

In the present study we measured circulating levels of the intracellular enzyme visfatin, hypothesized to be a marker of endothelial cell damage [62] .Compared to healthy controls, visfatin concentrations were found to be increased significantly in CKD patients compared to control group (Z=-3.224, p =0.001). When the different groups of patients were compared visfatin concentrations were found to be increased in all CKD stages except stage 1(group I) (χ2=21.59 , p =0.001). This result is in agreement with the result of previous recent studies.[63]In the present study a significant positive correlation was found between serum levels of visfatin and triglycerides, total cholesterol, LDL-cholesterol (r =0.453, p < 0.001;r=0.391, p =0.001;r = 0.463, p = < 0.001,respectively). This finding in accordance with **Mu J *et al.*** [64] who found similar results and gave the hypothesis that visfatin is associated with atherosclerosis in patients with CKD.

Visfatin also functions as a proinflammatory adipocytokine that is secreted by neutrophils in response to inflammatory stimuli and upregulates the production of cytokines in the monocytes. It was reported that visfatin promotes angiogenesis which can be associated with CKD-related vascular dysfunction [65] . Moreover visfatin was upregulated in the foam cell macrophages within human unstable carotid and coronary atherosclerotic lesions that play a role in plaque destabilization [66] . It is suggested that visfatin may play an important role in uremia-related atherosclerosis.

Kim *et al.* [67] investigated the effect of visfatin on vascular inflammation, a key step in a variety of vascular diseases. Visfatin induced leukocyte adhesion to endothelial cells and the aortic endothelium by induction of the cell adhesion molecules (CAMs); intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). Promoter analysis revealed that visfatin-mediated induction of CAMs is mainly regulated by nuclear factor-κB (NF-κB). Furthermore visfatin was found to stimulate ROS generation in endothelial cells. Based on visfatin enhanced NAD(P)H dependent ROS production in endothelial cells these observations, visfatin may be able to accelerate vascular diseases through ROS overproduction by inducing vascular damage. In addition it was demonstrated that antioxidants blocked visfatin-induced NF-κB activation and CAM expression [67,68] .

We extend this finding by showing an independent positive association between serum levels of visfatin and total nitrite and nitrate (r=0.475, p < 0.001). This observation may be due to the fact that a dysfunctional adipose tissue signaling-reflected by elevated visfatin in CKD may directly affect the vascular endothelium, causing its dysfunction. It appears likely that reduced glomerular filtration rate can contribute to the accumulation of adipocytokines. This could explain the marked dysmetabolism in chronic kidney disease. The kidney plays an important role in the regulation of adipokines, and altered renal handling of these substances might contribute to an increase in the uraemia-associated increased risk of cardiovascular disease and mortality [69] .

Recently, visfatin has been reported to be inversely correlated with functional changes in the endothelium as assessed by flow-mediated vasodilatation of the brachial artery in CKD and early diabetic nephropathy. Our results are supported by **Yilmaz *et al.*** [70] who documented that endothelial function improved during the first month after renal transplantation, and the degree of improvement correlated to reductions in circulating visfatin. In view of these results, serum visfatin is recommended as one of the most promising markers of ED in CKD patients.

Fetuin-A is a circulating protein mostly synthesized in the liver and ubiquitously present in the extracellular space [71]. Fetuin-A is known to be an important inhibitor of vascular and soft tissue calcification. It has been proposed as a protective agent by binding hydroxyapatite structures and causes solubilization of calcium phosphate salt [72].

In the present study serum fetuin-A concentrations were found to be significantly decreased in CKD patients compared to the control group (Z=-3.325, p =0.001), when different stages of

CKD patients were compared fetuin-A concentrations were found to be decreased in all CKD stages except stage 1 (group I) in comparison with the control group ($\chi 2=21.59$, $p=0.001$). Low serum fetuin-A concentrations were observed relatively early in CKD, beginning at stage 2 (group II). This observation indicates that vascular calcification and CVD are likely to develop early during the progression of CKD which is further substantiated by parallel development of endothelial dysfunction in these patients.

Given the importance of vascular calcification in the development of high rate of CVD, several investigators studied the role of fetuin-A in CKD, mostly in patients with advanced disease.[73] In a cross-sectional study, **Ketteler *et al.,*** [74] have shown that chronic hemodialysis patients had lower levels of fetuin- A concentrations than healthy controls. They found fetuin-A deficiency was associated with inflammation and mortality in patients on chronic hemodialysis. Also an Egyptian study was conducted in Cairo University by **El-Shehaby *et al.,*** [75] and revealed that serum fetuin-A was significantly lower in hemodialysis patients than the control subjects and a significant association between low levels of fetuin-A and high calcium score and valvular calcification was found.

To investigate the relationship between fetuin-A and a few of the mechanisms involved in the development of atherosclerosis, a significant negative correlation was found between serum levels of fetuin-A and serum levels of triglycerides, total cholesterol and LDL-cholesterol ($r = -0.310$; $p =0.008$,$r = -0.256$; $p =0.03$, $r = -0.420$; $p <0.001$; respectively), while a significant positive correlation was found between serum levels of fetuin-A and HDL-cholesterol ($r= 0.416$, $p <0.001$) in CKD patients.

In parallel with the previous results, in patients with metabolic syndrome, increased levels of fetuin-A were strongly associated with the components of the metabolic syndrome and with an atherogenic lipid profile [71] . Furthermore, **Stenvinkel *et al*.**, [76] reported that low fetuin-A levels were associated with malnutrition, inflammation and cardiovascular mortality. It was found that fetuin-A serum levels were significantly lower in CKD patients with calciphylaxis compared with other CKD subjects; in addition the inability of human uremic plasma to inhibit the precipitation of calcium and phosphorus is corrected by the addition of fetuin-A which accounts for more than 50% of the precipitation inhibitory effect of serum [77,78]. In support of these observations, **Huang *et al.***[79] found an inverse relationship between the extent of coronary calcification and endothelium-dependent flow mediated dilatation (FMD) in 124 patients with suspected coronary artery disease. These studies have led to the conclusion that there might be a biological link between vascular calcification which is associated with low levels of calcification inhibitors and derangement in endothelial function in CKD patients [79] .

This study also demonstrated the relationship between fetuin-A levels and endothelial dysfunction as there was a significant negative correlation between serum levels of fetuin-A and serum levels of total nitrate and nitrite and visfatin ($R=-0.264$; $p =0.025$,$r=-0.269$; $p =0.022$; respectively). A possible explanation for this correlation is that fetuin-A administration significantly inhibits TNF-α in an experimental model [80] and TNF-α suppressed visfatin gene expression in an *in vitro* study [16], in addition visfatin [81] and NO may be up regulated while fetuin-A is down regulated during the process of ED. [80] In contrast to this study **Uz *et al.,*** [81] failed to find a correlation between serum visfatin and serum fetuin – A.

Lower fetuin-A levels could contribute to endothelial dysfunction in CKD patients by several potential mechanisms. First, fetuin-A is a negative acute-phase reactant, given the importance of inflammation in endothelial dysfunction, low levels of fetuin-A might simply reflect an inflammatory condition. [38] Second, Calcification has been shown to be initiated with the release of membrane-bound vesicles; which normally contain local calcification inhibitors, from vascular smooth muscle cell (VSMC). Reduced levels of calcification inhibitors, such as fetuin-A, would lead to both vesicle and VSMC calcification [82]. **Cola *et al.,*** [83] have suggested that prolonged exposure of stressful stimuli to endothelial cells may lead to expression of genes that promote vascular calcification. It has also been shown that VSMC have a potential to transform to osteoblast- like cells [84].

Studies have reported relationships between fetuin-A and coronary artery score as well as carotid plaque in CKD patients; carotid intima-media thickness (CIMT) has been shown as a powerful predictor of cardiovascular events in the general population [85]. **Zoccali *et al* .,** [86] have shown that CIMT is a predictor of adverse cardiovascular events in patients on hemodialysis. **Caglar *et al.,***[38] suggested that fetuin-A levels might be another contributor for the development of CIMT supporting the potential role of fetuin-A in the development of accelerated atherosclerosis. Recent studies have demonstrated a relationship between vascular calcification and endothelial dysfunction, indicating a mechanistic relationship between endothelial dysfunction and vascular calcification, at least in part mediated through calcification inhibitors.[87] Given the importance of the role of fetuin- A in the development of calcification, one may suggest that low fetuin-A levels might contribute to endothelial dysfunction by

inducing calcification. Assessment of serum levels of fetuin-A may be of value to identify those subjects at higher risk of development and progression of vascular lesion and may be a novel therapeutic approach.

In order to clarify predictors of endothelial dysfunction in the CKD patients a stepwise regression model was done. Variables expected to influence the endothelial dysfunction represented as serum total nitrate and nitrite and the predictors were (the age, serum levels of triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, fetuin–A and visfatin) were included. According to this model the strongest predictors of endothelial dysfunction, evaluated as serum total nitrate and nitrite, were visfatin and HDL-cholesterol (beta= 0.635, p <0.001 and beta=-0.388, p =0.0039; respectively), the model demonstrated that 52% of the changes in serum total nitrate and nitrite could be explained and accounted by serum visfatin plus serum HDL-cholesterol and an equation was developed to estimate serum total nitrate and nitrite from knowing the values of serum visfatin and HDL-cholesterol. According to the results of this study, serum levels of visfatin and HDL-cholesterol were found to be the most important predictors of endothelial dysfunction.

While several studies [74,75], including this study, reported low fetuin-A levels in CKD patients, the mechanisms that result in decreased fetuin-A concentrations are not clear. In an attempt to know more about the pathogenesis of increased risk of ectopic calcification and cardiovascular events in patients with renal failure, restriction enzyme analysis was done for detection of restriction fragment length polymorphism of fetuin-A gene; (C→G);Thr256Ser at *SacI* recognition site known to be associated with a higher cardiovascular mortality risk in the hemodialysis population.

There was no statistically significant difference between CKD patients and the control group according to the frequencies of the three fetuin–A genotype polymorphisms (C→G);Thr256Ser (χ2=0.414, p =0.813) In both CKD patients and the control group, the distribution of the fetuin-A (C→G);Thr256Ser gene polymorphisms did not show statistically significant relationship between serum fetuin-A levels and the three genotypes in CKD and the control group (χ2=4.305; p =0.116, χ2=1.756; p =0.416; respectively). Consequently, neither altered (C→G); Thr256Ser polymorphism of the fetuin-A gene appears neither to affect serum fetuin-A levels nor to have prognostic effect for the progression to cardiovascular disease in this population.

It should be noted that to our knowledge there are only two previous studies [76,88] on the association of fetuin-A (C→G); Thr256Ser gene polymorphisms with serum levels of fetuin-A in CKD. **Stenvinkel *et al.*,** [76] demonstrated that Swedish dialysis patients with fetuin-A 256Ser/ Ser (allele G) had lower serum fetuin- A levels and associated with higher cardiovascular mortality rates. However, **Cozzolino *et al.*,** [88] found that: In both Italian hemodialysis patients and the control group, the distribution of the fetuin-A gene did not show significant association between low serum fetuin-A levels and fetuin-A (C→G); Thr256Ser gene polymorphisms had no statistically significant difference between distribution of fetuin-A (C→G);Thr256Ser gene polymorphisms in hemodialysis patients and the healthy controls was found.

Probably, the reason that some recent clinical results are different from our findings may be explained by the differences in races. Another reason could be contributed towards numerous factors that lead to marked arterial calcification observed in CKD patients: all the 'classic' risk factors for atherosclerosis plus 'uraemia-associated' risk factors, such as duration of dialysis, uraemic toxins, inflammation and increased serum levels of phosphate, calcium-phosphate product and parathyroid hormone .[89]Finally, other fetuin-A single nucleotide polymorphisms not yet known may affect its serum level, however fetuin-A remains an important protective factor against arterial calcification, even if its definitive role remains to be elucidated [87] .

Conclusion

In conclusion, the present study showed that:

- Visfatin and fetuin-A may be novel markers for endothelial dysfunction in chronic kidney disease patients, and may be help in diagnosis of early stages of CKD. Visfatin and fetuin-A may play an important role in uremia-related atherosclerosis. However, serum total nitrate and nitrite is the best one of the studied markers in diagnosis of early stages of CKD.
- The distribution of the fetuin-A (C→G); Thr256Ser gene polymorphisms does not affect serum fetuin-A levels.

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