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Studies in animals show that fibroblast growth factor (FGF)-23 interferes with vascular reactivity induced by the nitric oxide (NO) system. To investigate the relationship between circulating FGF-23 levels and the response of forearm blood flow to ischemia (flow-mediated vasodilatation, FMD) and nitroglycerin, we tested 183 patients with stage 3–4 chronic kidney disease (CKD). None of them had cardiovascular complications or were taking drugs interfering with vascular function. Patients with FGF-23 levels above the median had significantly lower glomerular filtration rate, FMD, and fetuin-A levels (an anti-inflammatory molecule and potent inhibitor of calcification). They also had higher proteinuria and phosphate levels when compared to patients whose FGF-23 levels were below the median. The response to nitroglycerin was not different between the two groups. Multiple regression analysis showed that the relationship between FGF-23 and FMD was only modestly sensitive to adjustment for classical risk factors, biomarkers of bone mineral metabolism, high-sensitivity C-reactive protein, and homeostatic model assessment index. Adjustment for asymmetrical dimethyl arginine (ADMA) weakened the strength of this link; however, it remained highly significant. There was no independent association between FGF-23 and nitroglycerin. Thus, attenuation of FMD by ADMA suggests that this endogenous inhibitor of NO synthase may, in part, mediate the vascular effects of FGF-23 in patients with CKD.

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Endothelial dysfunction has now emerged as a relevant risk factor for major cardiovascular events in the general population[1] and in chronic kidney disease (CKD) patients, from the predialysis stages of disease[2] to end-stage renal disease.[3] At variance with findings in the general population that identified classical risk factors as the major drivers for endothelial dysfunction,[4] studies in CKD and end-stage renal disease patients have shown that nontraditional risk factors[5] such as accumulation of endogenous inhibitors of nitric oxide (NO) synthase, high C-reactive protein (CRP), and some adipose tissue cytokines such as adiponectin and visfatin[6] and hemoglobin[7] are the major correlates of this alteration in patients with CKD. Interestingly, in CKD patients factors that regulate bone homeostasis such as fetuin-A and parathyroid hormone (PTH)[8] are associated with reduced hemodynamic response to ischemia in the forearm, a classical test of endothelial function. In contrast, it has been recently demonstrated that phosphate intake physiologically contributes to the regulation of flow-mediated vasoregulation in healthy, young individuals.[9] These observations are cognate to findings in end-stage renal disease patients showing robust, inverse associations between the response to this test and circulating 25 hydroxy-vitamin D (25OHVD) and 1,25 dihydroxy-vitamin D.[10] Collectively, these studies point to endothelium as a target for factors that regulate bone mineral metabolism.

Fibroblast growth factor 23 (FGF-23) is a primary regulator of renal phosphate excretion.[11] FGF-23 is inversely associated with the glomerular filtration rate (GFR),[12] a relationship underlying a fundamental mechanism for maintaining serum phosphate constancy during CKD progression. Such an adaptation may have deleterious trade-offs because, independently of serum phosphate, high FGF-23 signals a high risk of death in end-stage renal disease patients.[13] FGF-23 is expressed both in the myocardium and in the vascular system.[14] An association between FGF-23 and left ventricular hypertrophy[15] has recently been reported in CKD patients and in elderly people

in the general population.[16] In contrast, a large study in apparently healthy individuals with GFR >60 ml/min per 1.73 m^2 in the general population documented a monotonical, inverse association between FGF-23 and the forearm blood flow response to ischemia.[17] Whether alterations in circulating FGF-23 are implicated in vascular dysfunction in CKD is unknown. Given the peculiar risk factor profile of patients with CKD, the question appears of relevance for our understanding of the complex relationship between the bone–kidney axis and cardiovascular disease. With this background in mind, we investigated the relationship between plasma FGF-23 levels and the forearm blood flow response to ischemia in the forearm in a sizable series of incident stage 3–4 CKD patients. To avoid the confounding effect of classical risk factors and drug treatments on vascular function, a critical issue in diseased people,[4,18] we excluded from the study patients who were exposed to major factors that may modify vascular function and/or FGF-23 levels, namely, smokers and diabetic patients as well as patients with background cardiovascular events and those who were on angiotensin-converting enzyme inhibitors, angiotensin II blockers, statins, and vitamin D compounds.

RESULTS

The demographic, clinical, and biochemical characteristics of the study population, as categorized according to the median FGF-23 value (36.7 pg/dl), are given in Table 1.

Categorical analyses of FGF-23 and other risk factors

Patients in the category with higher FGF-23 levels had a lower GFR ($P=0.008$), L-arginine ($P=0.006$), and L-arginine/adjustment for asymmetrical dimethyl arginine (ADMA) ratio ($P=0.003$), but higher ADMA ($P=0.02$), proteinuria ($P=0.03$), and phosphate levels ($P=0.001$). On this categorical analysis, flow-mediated vasodilatation (FMD) ($P=0.01$) but not nitroglycerin-mediated vasodilatation (NMD) ($P=0.37$) was significantly less in patients with FGF-23 above than in those below the median value. Fetuin-A levels ($P=0.01$) were lower in patients with FGF-23 above the median. Plasma 25OHVD tended to be lower in patients with high FGF-23 but the difference was not significant ($P=0.12$). There were no differences for age, gender, body mass index, blood pressures, low-density lipoprotein-cholesterol, serum calcium and phosphate, PTH, hemoglobin, symmetrical dimethyl arginine, and high-sensitivity CRP levels of patients with FGF-23 above the median and those below the median.

Linear regression analyses of FGF-23 and other risk factors

To better characterize the categorical associations of serum FGF-23 and to identify the linear correlates of FMD, we first performed simple regression analyses between these variables and all variables listed in Table 1.

Data analysis with FGF-23 and FMD as continuous variables let emerge a close association between these two parameters (Figure 1). FMD ($r=-0.31$, $P<0.001$) while this

Table 1 | Demographic, hemodynamic, and biochemical data in nondiabetic CKD 3–4 patients categorized according to median FGF-23 value (36.7 pg/ml)

	CKD Patients (*n*=183)	<36.7 pg/dl (*n*=94)	⩾36.7 pg/dl (*n*=89)	*P*-value
Age (years)	45 ± 13	45 ± 13	44 ± 12	0.57
Male sex, *n* (%)	92 (49)	53 (43)	51 (55)	0.51
BMI (kg/m^2)	24.7 ± 2.7	24.8 ± 2.7	24.6 ± 2.7	0.66
Systolic pressure (mm Hg)	133 ± 9	134 ± 9	133 ± 9	0.34
Diastolic pressure (mmHg)	85 ± 4	85 ± 4	84 ± 4	0.26
Calcium (mg/dl)	8.3 ± 0.5	8.3 ± 0.5	8.4 ± 0.5	0.71
Phosphate (mg/dl)	4.9 ± 1.2	4.6 ± 0.9	5.3 ± 1.4	**0.001**
iPTH (pg/dl)	138.2 ± 26.6	140.1 ± 27.9	136.3 ± 24.9	0.30
25OHVD (nmol/l)	49.5 ± 10.2	51.1 ± 10.8	47.9 ± 9.4	0.12
LDL-cholesterol (mg/dl)	117 ± 17	118 ± 16	117 ± 17	0.88
Insulin (UI/L)	7.2 ± 1.4	7.3 ± 1.5	7.1 ± 1.3	0.79
HOMA index	1.6 ± 0.3	1.5 ± 0.4	1.6 ± 0.3	0.53
hsCRP (mg/l)	18 (6–34)	18 (6–32)	19 (8–34)	0.61
GFR (ml/min per 1.73m^2)	33 ± 13	36 ± 13	31 ± 12	**0.008**
Proteinuria (g/24 h)	1.5 ± 0.9	1.6 ± 0.9	1.3 ± 0.7	**0.03**
ADMA (μmol/l)	3.5 ± 1.1	3.3 ± 0.9	3.7 ± 1.1	**0.02**
SDMA (μmol/l)	2.9 ± 0.8	2.8 ± 0.8	2.9 ± 0.8	0.59
L-arginine (μmol/l)	81.3 ± 10.9	83.3 ± 9.4	79.2 ± 11.4	**0.006**
L-arginine/ADMA	24.9 ± 7.3	26.5 ± 6.9	23.3 ± 7.4	**0.003**
Fetuin-A (ng/ml)	0.28 ± 0.04	0.29 ± 0.04	0.27 ± 0.04	**0.01**
Uric acid (g/dl)	4.5 ± 1.1	4.3 ± 1.0	4.6 ± 1.2	0.17
Hb (g/dl)	11.5 ± 1.3	11.5 ± 1.5	11.5 ± 1.1	0.75
NMD (%)	12.7 ± 0.8	12.7 ± 0.8	12.6 ± 0.9	0.37
FMD (%)	6.8 ± 0.9	7.0 ± 0.9	6.6 ± 0.8	**0.01**

Abbreviations: ADMA, asymmetric dimethyl arginine; BMI, body mass index; CKD, chronic kidney disease; FGF, fibroblast growth factor; FMD, flow-mediated dilatation; GFR, glomerular filtration rate; Hb, hemoglobulin; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NMD, nitroglycerin-mediated vasodilatation; 25OHVD, 25 hydroxy-vitamin D; iPTH, intact parathyroid hormone.
Data are expressed as mean ± s.d., median (min–max), or as percent frequency, as appropriate.
Bold values: $P<0.05$, statistically significant; SDMA, symmetrical dimethyl arginine.

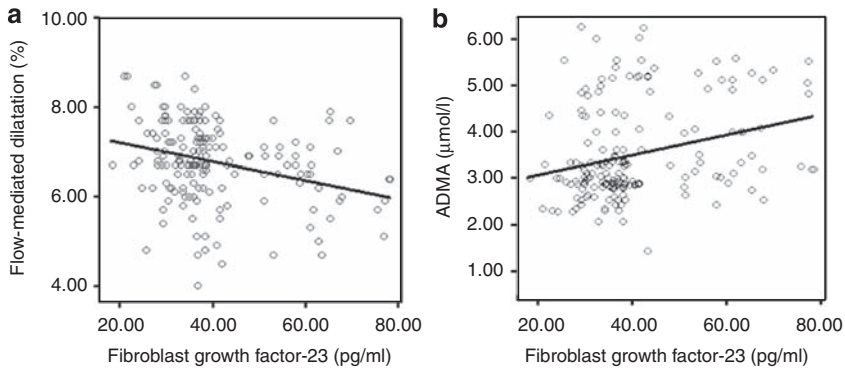

Figure 1 | The association between FGF-23 and endothelial dysfunction. Relationship between fibroblast growth factor (FGF)-23 and flow-mediated vasodilatation (FMD) (**a**) and asymmetrical dimethyl arginine (ADMA) (**b**).

hormone was only weakly related with NMD ($r=0.20$, $P=0.006$). FGF-23 was also directly related with ADMA ($r=0.28$, $P<0.001$) (Figure 1) and inversely associated with 25OHVD ($r=-0.30$, $P<0.001$), GFR ($r=-0.25$, $P=0.001$), L-arginine ($r=-0.21$, $P=0.004$), L-arginine/ADMA ($r=-0.33$, $P<0.001$), and fetuin-A ($r=-0.28$, $P<0.001$) levels.

Apart from being inversely associated with FGF-23, FMD correlated directly with the GFR ($r=0.27$, $P<0.001$) and fetuin-A ($r=0.36$, $P<0.001$), and inversely with ADMA ($r=-0.41$, $P<0.001$), symmetrical dimethyl arginine ($r=-0.19$, $P=0.009$), L-arginine/ADMA ($r=0.32$, $P<0.001$), 24 h proteinuria ($r=-0.16$, $P=0.04$), systolic blood pressure ($r=-0.38$, $P<0.001$), and high-sensitivity CRP ($r=-0.20$, $P=0.007$).

Multivariate analysis

To analyze the independent contribution of FGF-23 to the variance of FMD (Table 2), we constructed a series of multiple regression models based on traditional and nontraditional risk factors impacting upon this variable. In the unadjusted analysis, the FGF-23 and FMD levels were negatively correlated (Figure 1 and Table 2). Adjustment for the full set of Framingham risk factors did not produce any change in the correlation coefficient of the association. Further adjustment for indicators of renal function (GFR, proteinuria) and hemoglobin produced a moderate decrease (model 2: $\beta=-030$, model 3: $\beta=-023$, see Table 2) in the strength of the FGF-23–FMD correlation, which remained highly significant ($P<0.001$). Adjustment for emerging risk factors (ADMA, high-sensitivity CRP, homeostatic model assessment index, symmetrical dimethyl arginine, and L-arginine) substantially reduced the regression coefficient (model 3: $\beta=-0.15$, Table 2), which still remained highly significant ($P=0.009$). Further analysis showed that of the three emerging risk factors added to model 3 variables, ADMA was the sole variable responsible for the attenuation of the strength of the FGF-23–FMD association (Figure 2). This phenomenon suggests that this methylarginine is in the pathway, whereby high FGF-23 impairs endothelial function. Further adjustment for risk factors related with bone-mineral metabolism (calcium, phosphate, PTH, 25OHVD) did not modify the correlation coefficient of the FGF-23–FMD link (model 4: $\beta=-0.15$) (Table 2). The weak NMD–FGF-23 link was abolished after statistical adjustment (crude $\beta=-0.20$, $P=0.006$; adjusted $\beta=-0.09$, $P=0.24$).

DISCUSSION

This survey in patients with CKD shows that, independently of other traditional and nontraditional risk factors, high FGF-23 is associated with vascular dysfunction in this population.

Over the last decade a variety of experimental, clinical, and intervention studies documented that, in addition to hypertension and other classical risk factors, endothelial dysfunction, vascular calcification,[19] oxidative stress,[20] and inflammation[21] are risk factors of paramount importance in CKD patients. In previous studies we showed that ADMA, a NO synthase inhibitor, is implicated in endothelial dysfunction in CKD.[20–22] We also reported that vascular dysfunction in CKD is associated with low levels of fetuin-A,[8] an anti-inflammatory molecule and a potent inhibitor of the calcification process. This observation, which involved a factor considered as a primary in bone homeostasis, went along with findings indicating that vitamin D metabolites such as 25OHVD and calcitriol are strongly associated with FMD in stage 5D CKD patients.[10] Altogether, these data provided a rationale basis for the study of the bone–vascular connection in CKD and for planning mechanistic and intervention studies in this population.

FGF-23 has fully emerged as a central factor in the complex control of mineral metabolism.[11] Recent studies suggest that increased FGF-23 may contribute to the adverse outcomes in CKD because, independently of serum phosphate, it is associated with mortality,[13] left ventricular hypertrophy,[15] and progression of CKD.[12] FGF-23 is co-expressed with klotho in the vascular system and experiments performed in the klotho null mouse, a model characterized by extensive vascular calcification, strongly suggest that FGF-23 is an important factor in endothelial cell biology.[23] This mouse

Table 2 | Multiple regression models of FMD in CKD 3 and 4 patients

	Unadjusted (β, P)	Model 1 (β, P) (r^2=0.23)	Model 2 (β, P) (r^2=0.34)	Model 3 (β, P) (r^2=0.49)	Model 4 (β, P) (r^2=0.51)
FGF-23	**−0.30 (<0.001)**	**−0.30 (<0.001)**	**−0.23 (<0.001)**	**−0.15 (0.009)**	**−0.15 (0.007)**
Age (years)		−0.09 (0.25)	−0.08 (0.29)	−0.08 (0.29)	−0.08 (0.32)
Sex		0.05 (0.54)	0.01 (0.86)	0.01 (0.89)	−0.02 (0.83)
BMI		0.08 (0.30)	0.11 (0.16)	0.08 (0.30)	0.06 (0.46)
SBP		**−0.37 (<0.001)**	**−0.32 (<0.001)**	**−0.34 (<0.001)**	**−0.33 (<0.001)**
LDL-cholesterol		0.008 (0.92)	0.02 (0.76)	−0.06 (0.46)	−0.08 (0.31)
GFR			**0.27 (<0.001)**	**0.23 (<0.001)**	**0.22 (<0.001)**
Proteinuria			−0.13 (0.09)	−0.09 (0.22)	−0.06 (0.44)
Hemoglobin			**−0.23 (<0.001)**	**−0.28 (<0.001)**	**−0.28 (<0.001)**
HOMA				**0.18 (0.001)**	**0.19 (<0.001)**
hsCRP				−0.10 (0.18)	−0.12 (0.11)
ADMA				**−0.37 (<0.001)**	**−0.38 (<0.001)**
SDMA				−0.09 (0.23)	−0.11 (0.16)
L-arginine				−0.14 (0.07)	−0.14 (0.07)
Calcium					−0.03 (0.74)
Phosphate					0.09 (0.26)
PTH					**−0.13 (0.02)**
25OHVD					−0.04 (0.62)
Fetuin-A					−0.07 (0.38)

Abbreviations: ADMA, asymmetric dimethyl arginine; BMI, body mass index; CKD, chronic kidney disease; FGF, fibroblast growth factor; FMD, flow-mediated dilatation; GFR, glomerular filtration rate; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NMD, nitroglycerin-mediated vasodilatation; 25OHVD, 25 hydroxy-vitamin D; PTH, parathyroid hormone; SBP, systolic blood pressure; SDMA, symmetrical dimethyl arginine.
Bold values: $P<0.05$, statistically significant.

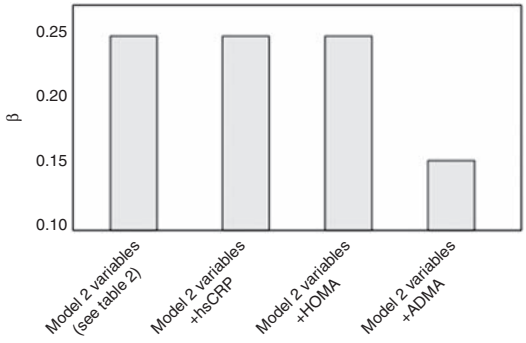

Figure 2 | Influence of adjustment of high-sensitivity C-reactive protein (hsCRP), homeostatic model assessment (HOMA) index, and asymmetrical dimethyl arginine (ADMA) on the correlation coefficient (β) of the fibroblast growth factor (FGF-23)–flow-mediated vasodilatation (FMD) relationship. The correlation coefficient was unaffected by hsCRP and HOMA but was substantially attenuated after adjustment for ADMA. Nonetheless, also after adjustment for ADMA, the FGF-23–FMD link remained highly significant ($P=0.009$).

has no FGF-23 activity because klotho is an obligatory co-receptor for FGF-23 functioning. The vasoactive response of aortic rings of klotho null mice to acetylcholine and noradrenaline is totally abolished.[24] Furthermore, elegant experiments of parabiosis demonstrated that endothelial function in the klotho null mouse can be fully restored by connecting the circulatory system of this mouse with that of the wild-type mouse.[25] The vascular response to klotho—FGF-23 is critically dependent on the NO system because no immunostaining for NO is found in the aorta of the klotho null mouse. More recent studies also documented that the FGF-23 null mouse has a biochemical phenotype almost identical to that of the klotho null mice and that also this knockout model undergoes severe vascular calcifications.[26]

Information on the putative interference of FGF-23 on vascular function in humans is very limited. At variance with the klotho null model, a recent population-based study[17] showed that high rather than low FGF-23 is associated with reduced vasoreactivity in individuals with normal renal function. Even though this apparently paradoxical association demands specific mechanistic studies, it is well known that other major vasoregulatory substances induce similar effects at very low and very high concentrations in *in vitro* models.[27] In the population-based study mentioned above,[17] the FGF-23–FMD relationship was confined to individuals with a GFR >60 ml/min, whereas no such an association was found in a subgroup of 208 patients with mild-to-moderate CKD. Furthermore, at variance with *in vitro* experiments that identify the endothelium as the critical layer involved in the response to klotho–FGF-23,[28] in this population-based study FGF-23 was inversely related with both the endothelium-dependent and independent vascular response implying that not only the endothelial cells but also the vascular smooth cells are involved in the response to FGF-23. In this study in a large series of stage 3 and 4 CKD patients with an average GFR of 33 ml/min, most of which with moderate-to-severe proteinuria, FGF-23 was coherently associated in an inverse manner with FMD both on categorical and noncategorical analyses. On the other hand, FGF-23 was unrelated with NMD on categorical analyses and only weakly related with this parameter on crude linear regression analysis, an association that was abolished by adjustment for other pertinent risk factors. These findings suggest that the vascular effects of FGF-23 in CKD patients are mainly mediated by the

endothelium. Even though directionally similar, the relationships we found in CKD differ from population-based data by Mirza *et al.*,[17] for three important aspects: first, in Mirza study FGF-23 was a correlate of vascular function only in individuals with normal renal function; second, in Mirza study the FGF-23–vascular function link involved both endothelium- and nonendothelium-mediated vasoregulation, whereas in our study this connection was largely confined to the endothelium-dependent component. Third, individuals studied by Mirza *et al.* were elderly (70 years) people with a relevant proportion (20%) of people with background cardiovascular comorbidities, whereas patients enrolled in our study were incident middle age and young patients (average age 45 years) who were specifically selected because of the absence of cardiovascular complications. Endothelium-independent (nitroglycerin or nitroprussiate mediated) vasoregulation is strongly influenced by age.[29,30] In the elderly, endothelium-independent vasoreactivity appears more closely related to cardiovascular risk factors than the endothelium-dependent responses.[29] Furthermore, although not reported, in Mirza study in the subgroup of 208 individuals with reduced GFR, CKD must have been of moderate degree, a possibility also suggested by the fact that vascular reactivity in these individuals was almost identical to that of individuals with normal renal function. Disturbed vasoregulation is indeed almost universal in patients with severely compromised renal function.

An intriguing finding in our study is that FGF-23 was directly associated with a biomarker of endothelial dysfunction similar to ADMA, an endogenous inhibitor of NO synthase. As mentioned, the effects of the klotho–FGF-23 complex in endothelial cell are mediated by the NO system because they are abolished by the inhibitors of NO synthase.[28] This biological connection is relevant for the interpretation of our data because we noted that ADMA attenuated the strength of the FGF-23–FMD link to a considerable extent, a phenomenon suggesting that FGF-23 and ADMA are in the same pathway that is conducive to vascular dysfunction, generating a hypothesis that can be tested in experimental studies. Yet, although much attenuated after adjustment for ADMA and other potential confounders, the FGF-23–FMD link remained robust and highly significant, an observation which implies that FGF-23 *per se* has a direct influence on endothelial function.

This study has limitations. First, the cross-sectional nature of our observations preclude cause–effect inferences about the FMD–FGF-23 link we observed. Second, we did not measure plasma 1,25 dihydroxy-vitamin D, a critical factor for mineral metabolism intimately connected with FGF-23, which has also been implicated in the control of vascular tone. However, this weakness is in part offset by the fact that we adjusted the analysis for 25OHVD, which is tightly associated with 1,25 dihydroxy-vitamin D levels in CKD patients.[31] Third, we did not perform an intervention to see whether modifications of FGF-23 levels induce changes in vascular reactivity. In this regard, it is worth noting that our cross-sectional data are in keeping with a recent clinical trial showing that a physiological oral load of phosphate, the main factor stimulating FGF-23 release, produces a clearcut decrease in FMD in young healthy individuals.[9]

In conclusion, FGF-23 is an independent correlate of vascular reactivity in CKD and ADMA in patients with CKD. The attenuation of the link between FGF-23 and FMD by ADMA suggests that this endogenous inhibitor of NO synthase may the pathway by which FGF-23 impairs vascular function in CKD patients.

MATERIALS AND METHODS

Patients

The Ethical Committee of Gulhane School of Medicine (Etlik-Ankara, Turkey) approved the study, and all patients gave their informed consent.

Between May 2005 and October 2009, 411 patients with CKD 3–4 were refereed to the Nephrology outpatient clinics of Gulhane School of Medicine. By protocol, we excluded diabetic patients, smokers, and patients with established atherosclerotic complication (coronary artery disease, congestive heart failure, or peripheral vascular disease). Patients who were being treated with angiotensin-converting enzyme inhibitors ($n=46$), angiotensin receptor blockers ($n=31$), statins ($n=21$), vitamin D ($n=18$), or taking polivitamins including folic acid and vitamin B12 ($n=14$) were also excluded. Overall, 183 nondiabetic patients, classified as KDOQI stage 3 ($n=90$) and stage 4 ($n=93$), could be enrolled into this study. None had acute infections at the time of the study. Protenuria was of mild degree (<1g/24 h) in 54 patients, of moderate degree (1–3g/24 h) in 118 patients, and of severe degree (>3g/24 h) in 11 patients.

The etiology of renal disease was as follows: glomerulonephritis ($n=42$), hypertensive nephropathy ($n=40$), Chronic pyelonephritis ($n=14$), reflux nephropathy ($n=10$), autosomal polycystic kidney disease ($n=18$), unknown ($n=59$). Twenty-one patients were on calcium channel blockers, 12 on α-blockers, and 7 on diuretics. Part of the data was published elsewhere.[7]

Laboratory measurements

All samples were obtained from patients in the morning after 12 h of fasting, for measurement of fasting plasma glucose, low-density lipoprotein-cholesterol, calcium and phosphate. Total plasma cholesterol and high-density lipoprotein-cholesterol were measured by enzymatic colorimetric method using Olympus AU 600 auto-analyzer with reagents from Olympus Diagnostics, GmbH (Hamburg, Germany). Low-density lipoprotein-cholesterol was calculated by Friedewald's formula.[32] Twenty-four hour urine collection was performed three times and the average of three 24 h proteinuria measurements was taken as representative of each participant 24 h protein excretion rate.

Patients were classified with respect to estimated GFR calculated according to the simplified version of the Modification of Diet, in Renal Disease formula as defined by Levey *et al.*[33]

The serum basal insulin value was determined by the coated tube method (DPC, Los Angeles, CA, USA). Homeostasis model assessment-insulin resistance (HOMA-IR) was computed by the following formula:[34] HOMA-IR = fasting plasma glucose (mg/dl) × immunoreactive insulin (μIU/ml)/405. Serum total calcium was measured by the cresolphtalein complex one method using Menagent Calcium 60sec kits (Menarini Diagnostics, Florence, Italy). Serum phosphorus

was measured by the ammonia molybdate complex method using Menagent Phosphofix kits (Menarini Diagnostics). For the measurement of high-sensitivity CRP, serum samples were diluted with a ratio of 1/101 with the diluents solution. Calibrators, kit controls, and serum samples were all added on each micro well with an incubation period of 30 min. After three washing intervals, 100 µl enzyme conjugate (peroxidase-labeled anti-CRP) was added on each micro well for additional 15 min incubation in room temperature in dark. The reaction was stopped with a stop solution and photometric measurement was performed at the 450 nm wavelength. The amount of serum samples was calculated as mg/l with a graphic that was made by noting the absorbance levels of the calibrators.

Serum FGF-23. Intact FGF-23 was measured using an enzyme-linked immunosorbent assay according to the manufacturer's protocol (Kainos Laboratories International, Tokyo, Japan). This second-generation, two-site, monoclonal antibody enzyme-linked immunosorbent assay has previously been shown to recognize the biologically active, intact FGF-23. The Kainos Intact FGF-23 assay has a lower limit of detection of 3 pg/ml and intra-assay and inter-assay coefficients of variation of less than 5%. The calculated overall intra-assay coefficient of variation was 2.5%, and the calculated overall inter-assay coefficient of variation was 2.8%. We measured all samples in duplicate.

ADMA, serum 25OH vitamin D3, PTH, fetuin-A. Measurement of serum ADMA, symmetrical dimethyl arginine, and L-arginine was done using high-performance liquid chromatography, as previously described into detail.[20,22] In brief, to 1 ml serum, 20 mg of 5-sulfosalisilic acid was added and the mixture was left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 2000 *g* for 10 min. Ten micro liters of the supernatant, which was filtered through a 0.2 µm filter, was mixed with 100 µl of derivatization reagent (prepared by dissolving 10 mg *o*-phtaldialdehyde in 0.5 ml of methanol, 2 ml of 0.4 M borate buffer (pH 10.0) and 30 µl of 2-mercaptoethanol) and then injected into the chromatographic system. Separation of ADMA was achieved with a 150 × 4 mm I.D. Nova-pak C18 column with a particle size of 5 µm (Waters, Millipore, Milford, MA, USA) using 50 mM sodium acetate (pH 6.8), methanol, and tetrahydrofurane as mobile phase (A, 82:17:1; B, 22:77:1) at a flowrate of 1.0 ml/min. The areas of peaks detected by the fluorescent detector (Ex: 338 nm; Em: 425 nm) were used as quantification. The variability of the method was less than 7%, and the detection limit of the assay was 0.01 µM.

To measure 25OHVD, we used high-performance liquid chromatography kits following manufacturer's instructions (ImmuChrom GmbH, Heppenheim, Germany. Quantification of 25-OH vitamin D3 was made by high-performance liquid chromatography system with UV (264 nm) detector (Thermo Electron, San Jose, CA, USA). The intra-assay coefficient of variation was 0.9–2.9%, and the calculated inter-assay coefficient of variation was 1.7–3.9% and recovery was 91%. Intact PTH was measured by IRMA, using a commercial kit (Immulite Intact PTH) from Diagnostic Product Corporation (Los Angeles, CA, USA) with a sensitivity of 1 pg/ml. Serum fetuin-A (AHSG) was measured by a Human fetuin-A ELISA kit (BioVendor Laboratory Medicine, Palackeho tr. 56, 612 00 Brno, Czech Republic) in an enzyme-linked immunosorbent assay plate reader (Synergy HT, Multidetection Multi Plate Reader, Bio-Tek Instruments, Highland Park, Winooski, VT, USA).

Vascular assessment

Arterial pressure was measured by a physician three times after a 15-min resting period in the morning, and mean values were calculated for systolic and diastolic pressures for all patients. Endothelium-dependent FMD and endothelium-independent vasodilatation (NMD) of the brachial artery was assessed noninvasively, using high-resolution ultrasound as described by Celermajer *et al.*[35] The method for the vascular assessment met the criteria which were mentioned by the International Brachial Artery Reactivity Task Force.[36]

Measurements were made by a single observer using an ATL 5000 ultrasound system (Advanced Technology Laboratories, Bothell, WA, USA) with a 12-Mhz prob. All vasoactive medications were withheld for 24 h before the procedure. The subjects remained at rest in the supine position for at least 15 min before the examination started. Subject's arm was comfortably immobilized in the extended position to allow consistent recording of the brachial artery 2–4 cm above the antecubital fossa. Three adjacent measurements of end-diastolic brachial artery diameter were made from single two-dimensional frames. All ultrasound images were recorded on S-VHS video cassette recorder (SVO-9500 MDP, Sony, Tokyo, Japan) for subsequent blinded analysis. A pneumatic tourniquet was inflated to 300 mm Hg with obliteration of the radial pulse. After 5 min, the cuff was deflated. Flow measurements were made 60 s post-deflation. After a further 15 min measurements were repeated, and again 3 min after administration of sublingual glyceryl trinitrate (400 µg). The maximum FMD and NMD diameters were calculated as the average of the three consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. The FMD and NMD were then calculated as the percent change in diameter compared with baseline resting diameters.

Statistical analysis

All the statistical analyses were performed by SPSS 11.0 (SPSS, Chicago, IL, USA) statistical package. Non-normally distributed variables were as median (range) and normally distributed variables were expressed as mean ± s.d. as appropriate. A *P*-value <0.05 was considered to be statistically significant. Differences among the groups were analyzed by Student t test. Standard correlation analysis was used for testing associations between paired variables. Finally, multiple regression analysis was applied to test the independent link between vascular function and potential functional correlates of this outcome variable. To this scope, we computed models of increasing complexity adjusting for traditional (Framingham risk factors) and emerging risk factors. Multivariate models were of adequate statistical power because included at least 10 observations for each covariate in the same models.

DISCLOSURE

All the authors declared no competing interests.

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