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Plasma endocan levels associate with inflammation, vascular abnormalities, cardiovascular events, and survival in chronic kidney disease

Mahmut I. Yilmaz[1], Dimitrie Siriopol[2], Mutlu Saglam[3], Yasemin G. Kurt[4], Hilmi U. Unal[1], Tayfun Eyileten[1], Mahmut Gok[1], Hakki Cetinkaya[1], Yusuf Oguz[1], Sebahattin Sari[3], Abdulgaffar Vural[1], Irina Mititiuc[2], Adrian Covic[2] and Mehmet Kanbay[5]

[1]Department of Nephrology, Gülhane School of Medicine, Ankara, Turkey; [2]Department of Nephrology, Gr.T.Popa University of Medicine and Pharmacy, Iasi, Romania; [3]Department of Radiology, Gülhane School of Medicine, Ankara, Turkey; [4]Department of Biochemistry, Gülhane School of Medicine, Ankara, Turkey and [5]Division of Nephrology, Department of Medicine, Istanbul Medeniyet University School of Medicine, Istanbul, Turkey

Plasma endocan levels are elevated in a large number of diseases, and may reflect endothelial cell dysfunction. There are currently no data on endocan in patients with chronic kidney disease (CKD). Therefore, we measured plasma endocan in 251 patients with CKD (stage 1–5) and 60 control individuals. Plasma endocan concentrations correlated with estimated glomerular filtration rate (eGFR), different markers of inflammation (pentraxin 3 and high-sensitivity C-reactive protein), and vascular abnormalities (flow-mediated vasodilation (FMV) and carotid intima media thickness (CIMT)). All-cause mortality and cardiovascular events (CVE) were also analyzed with respect to plasma endocan. Patients with CKD showed significantly increased plasma endocan (4.7 [IQR 1.9–9.4] compared with controls [IQR 1.1–1.5] ng/ml), with values progressively higher across stages of CKD. On univariate analysis, plasma endocan concentrations correlated negatively with eGFR and FMV, but positively with both markers of inflammation and CIMT. However, on multivariate analysis only high-sensitivity C-reactive protein, FMV, and CIMT remained significantly associated with plasma endocan. On Cox survival analysis, endocan levels were associated with all-cause mortality and CVE in these patients. Thus, plasma endocan increases in the presence of decreasing eGFR and influences all-cause mortality and CVE in patients with CKD independent of traditional and nontraditional risk factors.

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Correspondence: *Mehmet Kanbay, Department of Medicine, Division of Nephrology, Istanbul Medeniyet Universitesi, Goztepe Egitim ve Arastirma Hastanesi, Kadikoy, Istanbul 03490, Turkey. E-mail: drkanbay@yahoo.com*

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The prevalence of chronic kidney disease (CKD) has reached epidemic proportions, with ~10–13% of the population worldwide showing signs of this disease.[1] Cardiovascular disease (CVD) is the main cause of morbidity and mortality in CKD patients.[2] The process of cardiovascular damage starts very early during progression in well-defined CKD, long before the dialysis stage is reached,[3] which is important because individuals with CKD are more likely to die than to develop end-stage renal disease.[4] The connections between CKD and CVD are probably numerous,[5] with inflammation and endothelial dysfunction being the most important. Better understanding of the relationship between CKD and CVD is essential for guiding future strategies for screening and treatment.

Endocan, previously called endothelial cell-specific molecule-1, is a soluble proteoglycan of 50 kDa expressed by the vascular endothelium. Increasing experimental evidence has reported that endocan is overexpressed in cancer, sepsis, obesity, or inflammatory conditions[6] and is related to patients' outcome in different conditions such as sepsis[7] and cancer.[8–10] endocan may have roles in the vascular contribution to organ-specific inflammation and in endothelium-dependent pathological disorders and may represent a novel endothelial cell dysfunction marker.[7,11]

The aim of this study was threefold: (a) to evaluate endocan levels in CKD (vs. non-CKD) patients and their relation with GFR; (b) to investigate the relationship between endocan and different markers of inflammation and endothelial dysfunction in the same CKD population; and (c) to test the ability of endocan as a novel predictor of all-cause mortality and cardiovascular events (CVE) in CKD patients.

RESULTS

Main demographic, clinical, and biochemical parameters of the patients included in the study

A total of 251 patients (51% male; mean age = 45.8 ± 13.5 years) with CKD and 60 nondiabetic, non-CKD controls

(45% male) were evaluated in this study. The most frequent causes of CKD were diabetes mellitus (23.1%), hypertension (18.7%), and chronic glomerulonephritis (15.9%), but in a large proportion of patients (27.1%) the etiology was unknown. The mean arterial pressure was 135.5 ± 10.6/84.2 ± 4.4 mm Hg (Table 1). The demographic and clinical characteristics of the population across the different CKD stages are shown in Supplementary Table S1 online.

Patients in the control group were older and had lower diastolic and systolic blood pressure. Patients with CKD showed increased endocan (4.7 ng/ml [interquartile range (IR) 1.9–9.4 ng/ml] vs. 1.2 ng/ml [IR 1.1–1.5 ng/ml], $P<0.001$), hsCRP (11.0 mg/l [IR 7.0–22.0 mg/l] vs. 2.0 mg/l [IR 1.2–2.4 mg/l], $P<0.001$), and pentraxin 3 (PTX3) (4.9 ng/ml [IR 2.8–8.6 ng/ml] vs. 2.2 ng/ml [1.5–3.1 ng/ml], $P<0.001$) levels. The CKD patients had also higher carotid intima media thickness (CIMT) (0.7 mm [IR 0.6–0.8 mm] vs. 0.6 mm [0.6–0.6 mm], $P<0.001$) and reduced flow-mediated vasodilation (FMD) (7.0% [IR 6.1–7.5%] vs. 8.7% [8.1–9.2%], $P<0.001$) values compared with controls. Because of the nonmatched selection of the controls, we performed an additional analysis to compare endocan values between controls and the nondiabetic nonhypertensive CKD subpopulation ($N=165$ out of the total cohort). Even in this subgroup of CKD patients, we found significantly higher endocan values (3.5 ng/ml [IR 1.2–7.2 ng/ml], $P<0.001$) compared with non-CKD controls.

The median value of endocan was 4.71 ng/ml (IR 1.9–9.4 ng/ml), with values progressively higher across the CKD stages (see Supplementary Table S2 online and Figure 1). Higher endocan values were recorded in diabetic vs. nondiabetic patients (7.2 ng/ml [IR 3.2–12.5 ng/ml] vs. 3.7 ng/ml [IR 1.3–8.2 ng/ml], $P<0.001$), but there were no significant differences between genders or other CKD etiologies.

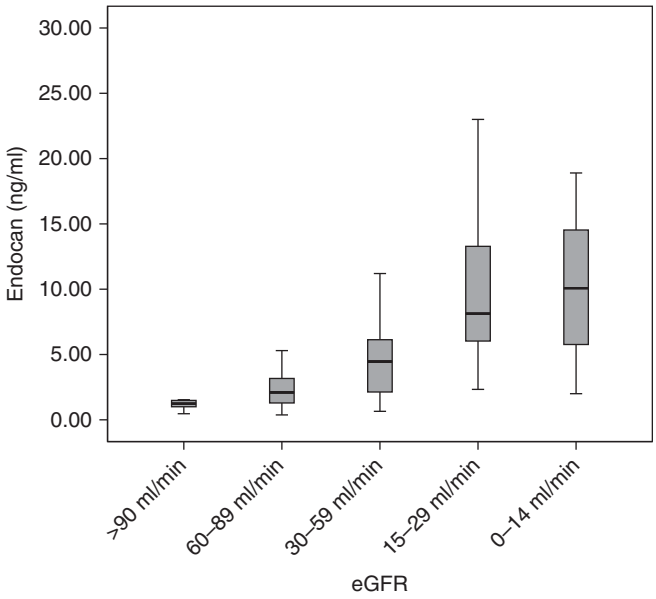


Figure 1 | Endocan values (ng/ml) in the control group and across the CKD population.

Table 1 | Demographic and clinical characteristics of the study population

	All ($n=251$)	1st quartile ($n=63$)	2nd quartile ($n=64$)	3rd quartile ($n=62$)	4th quartile ($n=62$)	P^a
Endocan, ng/ml	4.7 (1.9–9.4)	1.2 (0.9–1.3)	2.8 (2.1–3.7)	6.6 (5.5–7.8)	13.3 (10.9–15.8)	
Age, years	45.8 ± 13.5	47.1 ± 13.3	47.1 ± 13.7	44.6 ± 12.8	44.1 ± 14.3	0.45
Male, *n* (%)	128 (51.0)	31 (49.2)	30 (46.9)	34 (54.8)	33 (53.2)	0.80
BMI, kg/m^2	25.9 ± 2.7	26.6 ± 2.5	26.0 ± 2.7	25.6 ± 3.1	25.4 ± 2.4	0.06
Hypertension, *n* (%)	47 (18.7)	4 (6.3)	13 (20.3)	11 (17.7)	19 (30.6)	**0.01**
Systolic pressure, mm Hg	135.5 ± 10.6	133.9 ± 7.9	137.0 ± 9.6	133.3 ± 11.5	137.5 ± 12.6	0.10
Diastolic pressure, mm Hg	84.2 ± 4.4	83.4 ± 3.9	84.4 ± 4.1	83.2 ± 4.9	85.6 ± 4.1	**0.01**
Diabetes, %	58 (23.1)	3 (4.8)	15 (23.4)	17 (27.4)	23 (37.1)	**<0.001**
History of CVD, %	46 (18.3)	11 (17.5)	7 (10.9)	11 (17.7)	17 (27.4)	0.12
Cardiovascular episode	33 (13.1)	6 (9.5)	4 (6.3)	11 (17.7)	12 (19.4)	0.09
Stroke	6 (2.4)	2 (3.2)	1 (1.6)	0 (0)	3 (4.8)	0.34
PVD	6 (2.4)	3 (4.8)	1 (1.6)	0 (0)	2 (3.2)	0.40
Aortic aneurysm	1 (0.4)	0 (0)	1 (1.6)	0 (0)	0 (0)	1.00
Etiology of CKD, n (%)						
Diabetes	58 (23.1)	3 (4.8)	15 (23.4)	17 (27.4)	23 (37.1)	**<0.001**
Glomerulonephritis	40 (15.9)	15 (23.8)	7 (10.9)	11 (17.7)	7 (11.3)	0.15
Hypertension	47 (18.7)	4 (6.3)	13 (20.3)	11 (17.7)	19 (30.6)	**0.01**
ADPKD	10 (4.0)	3 (4.8)	1 (1.6)	3 (4.8)	3 (4.8)	0.73
Reflux nephropathy	12 (4.8)	5 (7.9)	2 (3.1)	4 (6.5)	1 (1.6)	0.32
Amyloidosis	16 (6.4)	8 (12.7)	2 (3.1)	4 (6.5)	2 (3.2)	0.09
Unknown	68 (27.1)	25 (39.7)	24 (37.5)	12 (19.4)	7 (11.3)	**<0.001**
Smoking, *n* (%)	112 (44.6)	24 (38.1)	24 (37.5)	28 (45.2)	36 (58.1)	0.07

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; BMI, body mass index; CKD, chronic kidney disease; CVD, cardiovascular disease; IR, interquartile range; PVD, peripheral vascular disease; SD, standard deviation.
Data are expressed as mean ± SD, median with IR, or percent frequency, as appropriate. Bold values are statistically significant.
[a]Comparison between groups.

Association of endocan levels with clinical, biochemical, and vascular markers

We divided the CKD patients into quartiles according to endocan values (<1.97; 1.97–4.71; 4.72–9.38; >9.38 ng/ml). Patients in the first quartile had a lower prevalence of diabetes (Table 1) and a higher eGFR (Table 2). There was also a significant trend between these groups with regard to hsCRP and PTX3 (as markers of inflammation), calcium, phosphate, and PTH (as markers of CKD—mineral and bone disorder), and CIMT and FMD (as markers of vascular abnormalities) (Table 2).

As shown in Table 3, simple linear regression analysis revealed that log endocan values were positively associated with log CIMT, log hsCRP, log PTX3, diabetes, hypertension, diastolic blood pressure, smoking, log proteinuria, log phosphate, and log intact parathyroid hormone, but negatively associated with log FMD, calcium log eGFR, low-density lipoprotein levels, and body mass index. In a multiple linear regression model, including all these predictors of endocan, only log FMD, CIMT, log hsCRP, serum calcium, diabetes, and smoking status maintained an independent association with endocan (adjusted R^2 of the model = 0.56, $P<0.001$). Using log glucose, as a continuous variable, instead of diabetes, as a categorical variable, and performing the same analysis did not affect the final model (Supplementary Table S3 online).

All-cause mortality and CVE according to endocan levels

During the follow-up period (median 42 months, IR = 36.0–45.0 months), 25 patients died (23 attributable to cardiovascular deaths), and 84 patients had fatal and nonfatal CVE.

In univariate Cox survival analysis (Table 4), increasing endocan levels were associated with an elevated risk of death (a threefold increase for each increment of 1 s.d. in log endocan values) and CVE (a 2.75-fold increase for each

Table 3 | Determinants of endocan levels (log endocan) in CKD patients ($N = 251$)

	Simple linear regression			Backward stepwise multiple regression		
Variables	Coefficient	SE	P	Coefficient	SE	P
Log FMD, %	−3.545	0.260	<0.001	−1.739	0.313	<0.001
Log CIMT, mm	3.202	0.271	<0.001	1.166	0.308	<0.001
Log hsCRP, mg/l	0.643	0.067	<0.001	0.216	0.068	0.01
Calcium, mg/dl	−0.370	0.041	<0.001	−0.109	0.038	0.01
Diabetes (Yes = 1)	0.302	0.063	<0.001	0.207	0.045	<0.001
Smoking (Yes = 1)	0.125	0.055	0.03	0.083	0.037	0.03
Log eGFR, ml/min/1.73 m^2	−0.491	0.043	<0.001			
Log proteinuria, g/day	0.442	0.124	<0.001			
Log PTX3, ng/ml	0.624	0.070	<0.001			
Hypertension (Yes-1)	0.210	0.070	0.01			
DBP, mm Hg	0.014	0.006	0.02			
LDL, mg/dl	−0.004	0.002	0.04			
BMI, kg/m^2	−0.028	0.010	0.01			
Log phosphate, mg/dl	1.954	0.211	<0.001			
Log iPTH, pg/ml	0.571	0.062	<0.001			

Abbreviations: BMI, body mass index; CIMT, carotid intima media thickness; CKD, chronic kidney disease; FMD, flow-mediated vasodilation; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; iPTH, intact parathyroid hormone; LDL, low-density lipoprotein; PTX3, pentraxin 3.

Table 2 | Biochemical parameters and vascular assessments of the study population

	All ($n = 251$)	1st quartile ($n = 63$)	2nd quartile ($n = 64$)	3rd quartile ($n = 62$)	4th quartile ($n = 62$)	P[a]
Endocan, ng/ml	4.7 (1.9–9.4)	1.2 (0.9–1.3)	2.8 (2.1–3.7)	6.6 (5.5–7.8)	13.3 (10.9–15.8)	
GFR (ml/min/1.73 m^2)	44.0 (16.0–73.0)	92.0 (68.0–98.0)	57.0 (31.0–68.8)	24.0 (15.8–56.3)	16.0 (4.0–22.3)	**<0.001**
CKD stage, *n* (%)	46 (18.3)	36 (57.1)	4 (6.3)	3 (4.8)	3 (4.8)	
	54 (21.5)	20 (31.7)	24 (37.5)	9 (14.5)	1 (1.6)	
	50 (19.9)	7 (11.1)	21 (32.8)	15 (24.2)	7 (11.3)	**<0.001**
	51 (20.3)	0 (0)	6 (9.4)	21 (33.9)	24 (38.7)	
	50 (19.9)	0 (0)	9 (14.1)	14 (22.6)	27 (43.5)	
Hemoglobin, g/dl	12.4 ± 2.2	12.7 ± 2.3	12.3 ± 1.9	12.4 ± 2.2	12.3 ± 2.2	0.68
Proteinuria, g/day	1.7 (1.2–2.1)	1.6 (0.9–1.9)	1.6 (1.1–1.9)	1.7 (1.3–2.1)	1.8 (1.6–2.5)	**0.04**
hsCRP, mg/l	11.0 (7.0–22.0)	6.0 (4.0–8.0)	10.0 (8.0–17.0)	16.5 (9.0–22.0)	24.8 (17.0–30.2)	**<0.001**
PTX3, ng/ml	4.9 (2.8–8.6)	2.9 (2.2–4.2)	4.8 (2.7–8.3)	6.7 (3.9–9.7)	8.1 (4.9–15.9)	**<0.001**
Total cholesterol, mg/dl	199.2 ± 17.7	199.1 ± 16.7	199.1 ± 16.3	199.2 ± 19.4	199.3 ± 18.9	1.00
Triglyceride, mg/dl	140.1 ± 15.1	139.6 ± 12.9	140.2 ± 15.0	139.4 ± 14.7	141.4 ± 17.7	0.63
LDL cholesterol, mg/dl	122.9 ± 16.5	123.9 ± 16.9	125.4 ± 15.9	122.9 ± 14.3	119.0 ± 18.2	0.17
HDL cholesterol, mg/dl	42.9 ± 5.6	43.7 ± 5.4	42.9 ± 5.7	41.7 ± 5.2	43.0 ± 5.9	0.17
Glucose, mg/dl	90.0 (80.0–101.0)	82.0 (80.0–92.0)	91.0 (81.0–100.0)	91.0 (80.0–118.3)	96.0 (87.8–145.8)	**<0.001**
Albumin, g/dl	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.3	0.83
25OHVD, nmol/l	51.3 ± 11.5	50.6 ± 10.1	52.2 ± 12.9	50.5 ± 12.3	51.7 ± 10.8	0.80
Calcium, mg/dl	8.6 ± 0.6	9.1 ± 0.6	8.7 ± 0.6	8.4 ± 0.5	8.3 ± 0.4	**<0.001**
Phosphate, mg/dl	4.8 (4.2–5.8)	4.2 (3.8–4.5)	4.8 (4.1–5.8)	5.4 (4.8–6.7)	5.4 (4.8–7.7)	**<0.001**
PTH, pg/ml	108.0 (50.0–157.0)	42.0 (31.0–60.0)	99.5 (52.3–138.8)	129.0 (98.8–169.3)	169.0 (134.5–235.8)	**<0.001**
FMD, %	7.0 (6.1–7.5)	8.1 (7.3–8.7)	7.2 (6.5–7.4)	6.4 (6.0–7.2)	6.0 (5.2–6.4)	**<0.001**
CIMT, mm	0.70 (0.60–0.82)	0.59 (0.55–0.65)	0.69 (0.60–0.76)	0.79 (0.69–0.84)	0.82 (0.75–0.90)	**<0.001**

Abbreviations: 25OHVD, 25 Hydroxyvitamin D; CIMT, carotid intima media thickness; CKD, chronic kidney disease; FMD, flow-mediated vasodilation; GFR, glomerular filtration rate; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; PTH, parathyroid hormone; PTX3, pentraxin 3.
Data are expressed as mean ± SD, or median with IR, as appropriate. Bold values are statistically significant.
[a]Comparison between groups.

increment of 1 s.d. in log endocan values). In multivariable Cox models, after adjustment for traditional (Model 1) and renal-specific risk factors (Model 2) endocan levels maintained an independent association with both outcomes (Table 4). In the final analysis (Model 3), an increase of 1 s.d. in log endocan levels was associated with a 2.38- and 3.54-fold augmentation in the risk for all-cause mortality and CVE, respectively. Using log glucose in place of diabetes did not have an influence on the model fit or on the final results of the analysis (Supplementary Table S4 online). In line with these results, Figure 2 shows the cumulative incidence of death and CVE according to the endocan quartiles.

Endocan—better predictive power?

Another central point of our investigation was to find out whether endocan has a predictive power beyond that of the usual risk factors.

Using receiver operating curve analysis, endocan had an area under the receiver operating curve for survival of 0.774 (95% confidence interval (CI) 0.717–0.824, $P<0.001$), which was larger than that of eGFR (0.626, 95% CI 0.563–0.685, vs. endocan: $z=3.64$, $P<0.001$), proteinuria (0.647, 95% CI 0.585–0.706, vs. endocan: $z=1.78$, $P=0.08$), FMD (0.732, 95% CI 0.672–0.785, vs. endocan: $z=0.89$, $P=0.37$), CIMT (0.731, 95% CI 0.671–0.785, vs. endocan: $z=1.00$, $P=0.32$), hsCRP (0.694, 95% CI 0.633–0.751, vs. endocan: $z=1.56$, $P=0.12$), and PTX3 (0.753, 95% CI 0.695–0.805, vs. endocan: $z=0.38$, $P=0.70$). The discriminative power for predicting CVE was also significantly higher for endocan (area under the corresponding receiver operating characteristic curve (AUROC) 0.851, 95% CI 0.800–0.892, $P<0.001$) than for all of the already mentioned variables ($P<0.05$).

Adding endocan to a prediction model based on traditional (age, gender, smoking status, diabetes, systolic blood pressure, HDL, and total cholesterol) and renal-specific risk factors (eGFR, proteinuria, and hsCRP) did not add much discrimination ability for all-cause mortality (model with endocan AUROC = 0.89 vs. model without endocan AUROC = 0.87; $P=0.19$). The total net reclassification improvement of 3% was statistically nonsignificant. In contrast, when analyzing CVE, adding endocan improved the model prediction capability (model with endocan AUROC = 0.93 vs. model without endocan: AUROC = 0.85; $P<0.001$). As for the second model, the total net reclassification improvement of 43% and both the 'event' and the 'non-event' net reclassification improvement were statistically significant ($P<0.001$ for all).

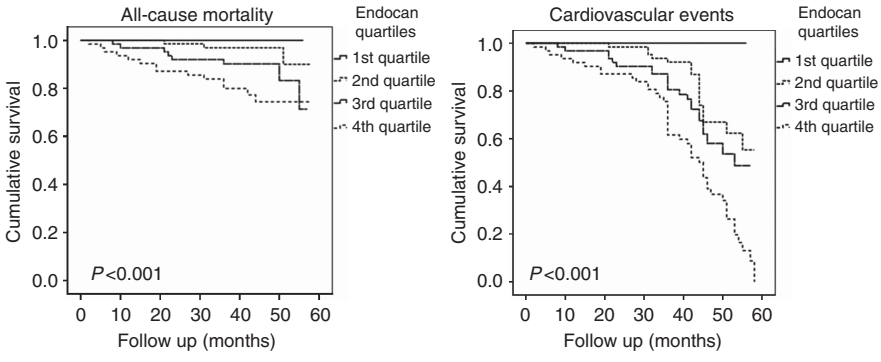
Table 4 | Endocan as a predictor of all-cause mortality and cardiovascular events

	All-cause mortality		Cardiovascular events	
	HR[a]	95% CI	HR[a]	95% CI
Unadjusted	3.02	1.75–5.19	2.75	2.06–3.68
Adjusted				
Model 1	2.78	1.54–5.04	2.44	1.78–3.33
Model 2	3.08	1.58–5.99	3.90	2.78–5.49
Model 3	2.38	1.17–4.82	3.54	2.36–5.29

Abbreviations: CI, confidence interval; HR, hazard ratio.
Model 1: age, gender, smoking status, diabetes, systolic blood pressure, high-density lipoprotein, and total cholesterol. Model 2: estimated glomerular filtration rate, proteinuria, and high-sensitivity C-reactive protein. Model 3: variables in model 1 + variables in model 2.
[a]Hazard ratio for each increase in 1 SD in log endocan.

DISCUSSION

The present study shows that endocan levels are inversely correlated with eGFR and that its value influences, independently of traditional and nontraditional risk factors, all-cause mortality and CVE in the CKD population. Importantly, adding endocan to a prediction model based on common and nontraditional risk factors improved the model prediction capability for fatal and nonfatal CVE. To our knowledge, this is the first study that evaluates endocan levels in this population and its impact on hard end points.



No at risk							
1st quartile	63	63	62	61	43	9	0
2nd quartile	64	63	63	62	37	14	1
3rd quartile	62	61	60	56	40	13	0
4th quartile	62	58	54	52	32	14	2

No at risk							
1st quartile	63	63	62	61	43	9	0
2nd quartile	64	63	63	62	37	14	1
3rd quartile	62	61	60	56	40	13	0
4th quartile	62	58	54	52	32	14	2

Figure 2 | Kaplan–Meier analysis for all-cause mortality and cardiovascular events (CVE) according to endocan quartiles.

Vascular inflammation is an important process in the development of CVD, and patients with CKD represent a subset with a higher risk of CVD.[12,13] Multiple possible explanations exist for the association between CKD and increased risks of death and CVD. Reduced kidney function is associated, among others, with increased levels of inflammatory factors and endothelial dysfunction.[2] Identifying biomarkers that would serve as indicators of widespread inflammation or as surrogates for endothelial cell activation and/or dysfunction is of particular importance.[7]

An initial step in vascular inflammation leading to atherogenesis is the adhesion cascade that involves the rolling, tethering, adherence, and subsequent transmigration of leukocytes through the endothelium. Recruitment and accumulation of leukocytes to the endothelium is mediated by an upregulation of adhesion molecules such as vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1.[14] Recently, it was shown that the serum levels of these soluble adhesion molecules are increased in patients with CKD,[15,16] probably through a reactive oxygen species-induced activation of nuclear factor-kappaB in vascular endothelial cells.[17] endocan affects the interaction of intracellular cell adhesion molecule-1/LFA-1 and may be implicated in the regulation of leukocyte extravasation at the inflammatory sites.[18] Abid *et al.*[19] have shown that endocan expression is mediated through a VEGF-dependent signaling pathway. Microenvironments that are rich in VEGF, such as CKD,[20] may activate both PKC-NF-κB and PI3KAKT-forkhead pathways in the endothelium, resulting in net induction of endocan gene expression.[21]

The FMD test, the standard noninvasive tool used to assess endothelial function,[18] and CIMT, a well-established surrogate of atherosclerosis, have been noted for their capacity to predict future CVE, in both non-CKD[22–24] and CKD populations.[12,25] Our study is in line with previous ones that showed increased CIMT and reduced FMD in CKD patients.[26–28] endocan levels were correlated with both CIMT and FMD values even after multiple adjustments, suggesting that in CKD patients endocan levels could be a useful marker of vascular disturbances. Quite recently, Oltean *et al.*[29] have shown that in brain-dead donors endocan levels are associated with resistin concentrations. Resistin is able to promote the endothelial cell activation and mount a robust proinflammatory response[21] through the NF-κB pathway.[30,31] The endothelium is a prime site of brain death–induced organ injury,[32] and the observed association between these two biomarkers strengthens the use of endocan as a surrogate for endothelial injury.

CRP and PTX3 are members of the family of pentraxins, which are small pentameric innate immunity effector proteins, and both can predict mortality[17,33–35] and CVE in a CKD population.[36,37] In agreement with previous studies,[20,24,38] the levels of this inflammatory markers are markedly increased in our patients with CKD. Both were associated with endocan values, but only high-sensitivity C-reactive protein (hsCRP) maintained this association in multivariate regression. This correlation was also revealed in other pathological conditions associated with systemic inflammation, such as Behcet disease[39] or even newly diagnosed hypertension.[30] In contrast to short pentraxins (such as CRP), PTX3 is produced at the actual site of inflammation,[40] suggesting that in CKD patients increased endocan levels could also be secondary to diffuse inflammation.

This is the first study that evaluates the relationship between endocan and eGFR. It has been demonstrated that endocan expression is highest in the kidneys, localized mainly in the peritubular cells of the medulla and cortex.[11,21] Whether the increased levels seen in our study are the result of an increased secretion or a reduced renal clearance is, at this moment, not known. The fact that its levels are increased in a variety of inflammatory diseases without renal involvement could reinforce the first of these two hypotheses. In addition, we have shown that its predictive capabilities go beyond eGFR and proteinuria, and this could suggest that endocan is more than simply a marker of renal function.

Our study had limitations and strengths. Because of the observational nature of our study, no cause–effect inferences about the relationship between the endocan levels and all-cause mortality or CVE in CKD patients could be made. However, this is a large study with a significant follow-up period and a significant number of events.

Another issue regarding our study is the generalization of our final results. Because of the nature of the investigation, we excluded all patients who were on any medication that could have influenced the endothelial function. Thus, we excluded patients who were taking ACEI/angiotensin receptor blockers or statins, which are now considered cornerstones in the management of CKD patients in general, and of diabetics in particular. However, all the other patients' characteristics that could have influenced survival were considered in detail and analyzed in different statistical models.

CONCLUSIONS

To the best of our knowledge, this is the first study that shows that serum endocan is associated with survival and fatal and nonfatal CVE in a nondialysis CKD population, independently of usually used markers of inflammation and vascular abnormalities. Further studies are needed to determine whether endocan is only a marker of a negative prognosis in these patients or whether it has an active role in the CKD–inflammation–endothelial dysfunction interconnection.

MATERIALS AND METHODS

Patients and study design

Between January 2007 and March 2013, 1347 patients were referred to the Renal Unit of the Gulhane School of Medicine Medical Center, Ankara, Turkey, for the first time because of suspected or manifest CKD.[41] Some of these patients were part of an ongoing prospective study, which is previously described in more detail.[12] All patients included in the study were diagnosed as having CKD according to the National Kidney Foundation K/DOQI Guidelines.[42] By protocol, and in order to minimize any confounding effects of

conditions that may influence endothelial dysfunction, 921 patients who were taking drugs that could influence endothelial function were excluded, including angiotensin-converting enzyme inhibitors ($n = 294$), angiotensin receptor blockers ($n = 288$), statins ($n = 144$), erythropoetin ($n = 116$), or supplemental vitamin pills ($n = 79$). Other exclusion criteria including acute infections and unwillingness to participate in the study were applied ($n = 88$). In addition, 87 eligible patients withdrew consent. Stages of CKD were determined using estimated glomerular filtration rates (eGFR), which were calculated via the modification of diet in renal disease equation.[43] A total of 251 patients were included in the final analysis. None of the patients in stage 5 CKD were on hemodialysis or peritoneal dialysis. The control group consisted of 60 patients from our outpatient department whose primary reason for referral was not diabetes, renal, or cardiovascular disease. Those patients were recruited solely on the basis of the absence of the aforementioned pathologies and not matched to the CKD cohort in terms of gender or age.

All included patients were followed-up for time-to-event analysis until occurrence of fatal or nonfatal CVE. Information on fatal and nonfatal CVE including death, stroke, and myocardial infarction were obtained from the Gulhane School of Medicine Medical Center registries. Hospital records and death certificates were reviewed by three of the investigators who were unaware of baseline parameters. If information could not be obtained, the patient was assumed to be lost to follow-up starting from the date of the last actual visit. Local ethics committee approved the study protocol, and all patients were included in the study after signing informed consent forms.

Biochemical analyses

All blood samples were obtained from patients in the morning after 12 h of fasting, for measurement of serum albumin, total serum cholesterol, triglyceride, high-density lipoprotein (HDL), and low-density lipoprotein cholesterol. Proteinuria was quantified using 24-hour timed urine collection. Intact parathyroid hormone was measured by IRMA, by using a commercial kit (Immulite Intact PTH) from Diagnostic Product Corporation (Los Angeles, CA) with a sensitivity of 1 pg/ml.

Serum human PTX3 measurements

Plasma PTX3 concentration was measured a posteriori from frozen samples by using a commercially available enzyme-linked immunosorbent assay kit (Perseus Proteomics, Tokyo, Japan). Description of these results has been published elsewhere.[44]

Serum endocan measurements

The concentration of human endothelial cell-specific molecule-1 was analyzed by ELISA using commercial kits (Aviscera Bioscience, Santa Clara, CA), in accordance with the manufacturers' instructions. Intra-assay coefficient of variation of endothelial cell-specific molecule-1 assay ranged from 6 to 8%, whereas inter-assay coefficient of variation ranged from 10 to 12%. The lower limit of detection for endothelial cell-specific molecule-1 was 98 pg/ml. Measurements were carried out using enzyme-linked immunosorbent assay plate reader Bio-Tek Synergy HT (Biotek Instruments, Winooski, VT). All the samples were measured in duplicate.

Assessment of endothelial function

Endothelial dysfunction was assessed according to the method described by Celemajer *et al.*[45] Measurements were made by a single observer using an ATL 5000 ultrasound system (Advanced Technology Laboratories, Bothell, WA) with a 12-Mhz probe. The vascular assessment method was in agreement with the criteria set forth by the International Brachial Artery Reactivity Task Force.[46] 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. Each subject's right 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 2D frames. All ultrasound images were recorded on Super Video Home System videotape for subsequent blinded analysis. The maximum FMD diameters were calculated as the average of the three consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. The FMD was then calculated as the percentage change in diameter compared with baseline resting diameters.

Assessment of intima media thickness

CIMT was assessed in all subjects. Briefly, a high-resolution B-mode ultrasound of the common carotid arteries with scanning of the longitudinal axis until the bifurcation and of the transversal axis was performed using an instrument generating a wide-band ultrasonic pulse with a middle frequency of 12 MHz (ATL 5000; Advanced Technology Laboratories). For each carotid artery, two longitudinal measurements were obtained by rotating the vessels at 180° increments along their axis. All patients and controls were blindly examined by one experienced operator (the intra-operator variability was 4%). IMT was measured at 1 cm proximal to the bifurcation on each side.

Statistical analysis

All statistical analyses were performed with SPSS 19.0 (SPSS, Chicago, IL) and MedCalc 12.5.0.0 statistical packages. Non-normally distributed variables were expressed as median with IR and normally distributed variables were expressed as mean ± s.d., as appropriate. Logarithmic conversion was performed for non-normally distributed variables, including endocan (log endocan), eGFR (log eGFR), proteinuria (log proteinuria), glucose (log glucose), FMD (log FMD), CIMT (log CIMT), hsCRP (log hsCRP), PTX3 (log PTX3), phosporus (log phosphorus), and log intact parathyroid hormone.

Among patients, comparisons were made using the paired *t*-test (normally distributed data) or the Wilcoxon signed-rank test (non-normally distributed data). A *P*-value < 0.05 was considered to be statistically significant. Between-group comparisons were assessed for nominal variables with the χ^2 test, and by Kruskal–Wallis test or one-way analysis of variance for the remaining variables. We applied a simple linear regression analysis to identify factors associated with log endocan. Stepwise multivariate regression analysis including all univariate associates ($P < 0.05$) was used to assess the predictors for endocan.

Survival and time-to-event analyses of cardiovascular outcomes were performed using Kaplan–Meier and Cox proportional hazards models, initially only with endocan, and subsequently adjusting for several groups of covariates. In model 1, we adjusted for traditional cardiovascular risk factors: age, gender, smoking status, diabetes, systolic blood pressure, HDL, and total cholesterol. In model 2, we adjusted for renal-specific cardiovascular risk factors: eGFR, proteinuria, and hsCRP. In model 3, we adjusted for variables used

in the previous two models. Data are presented in the form of hazard ratios and 95% CI. To avoid the problem of overfitting owing to the low number of incident outcomes, we performed bootstrapping validation, in order to determine the CIs for estimating β in the Cox proportional hazard regression.

Proportional hazards assumptions were tested using log minus log survival plots, and Schoenfeld residuals

The diagnostic accuracy was expressed as the AUROC, and the respective areas under the curves were calculated with 95% CI. The different AUROCs were compared using the method described by Hanley and McNeil.[47] The discriminative value of the endocan was further examined with the net reclassification improvement method.[48]

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Demographic and clinical characteristics of the study population according to CKD stages.
Table S2. Biochemical parameters and vascular assessments of the study population according to CKD stages.
Table S3. Determinants of endocan levels (log endocan) in CKD patients ($N = 251$) adjusting for glucose (log glucose) levels.
Table S4. endocan as predictor of all-cause mortality and cardiovascular events in adjusted for glucose (log glucose) models.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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