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Arterial calcification in patients with chronic kidney disease

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Abstract

Objective. In patients with chronic kidney disease (CKD), aortic calcification is more frequent and severe and it is also predictive of adverse cardiovascular outcome. The aim of the present study was to characterize aortic calcification in renal compared with non-renal patients.

Methods. Aortas of 31 patients with advanced CKD and of 31 age-and gender-matched controls were obtained at autopsy. Calcium and phosphorus content in the aorta was quantitated using x-ray analysis. The expression of calcification-promoting and calcification-inhibiting proteins was assessed using immunohistochemistry.

Results. The calcium and phosphorus content of the aorta was higher in CKD patients than in controls. Even in non-calcified aortic specimens of CKD, staining for Msx-2, BMP-2, bone sialo-protein, TNF-α and nitrotyrosine was significantly more marked compared to controls. The same proteins were immunodetected in calcified aortic specimens of both CKD and controls. In contrast, staining for transglutaminase-2 and Fetuin A was significantly reduced in CKD. Higher expression of cbfa-1 and Pit-1 was observed in all calcified aortas with no difference between CKD and controls. The expression of TNF-α, phospho-p38 and Msx-2 was correlated to the intensity of up-regulation of BMP-2 and osteoblastic transdifferentiation by VSMC even in non-calcified areas of the aortas of CKD.

Conclusion. The expression of markers characteristic for calcification is not different in calcified aorta of CKD patients compared to controls, but in CKD patients, evidence of inflammation, transformation to an osteoblastic phenotype and reduced expression of transglutaminase are also found even in non-calcified aorta.

Keywords: arterial calcification; cbfa-1; chronic kidney disease; osteoblastic transdifferentiation; TNF-α

Introduction

In individuals with chronic kidney disease (CKD), the risk of death from cardiovascular (CV) causes is substantially elevated and even greater than the risk of progression to end-stage renal disease [1]. In dialyzed patients, CV mortality is 15–30 times higher compared with the general population [2,3], but the CV risk is increased not only in end-stage CKD, but even in the earliest stages of CKD [4].

CKD is characterized by accelerated atherosclerosis and particularly by more pronounced vascular calcification [5–7]. Both progressive circumscript calcification of atherosclerotic plaques and diffuse calcification of the media of central arteries (Mönckeberg sclerosis) are observed. Calcified atherosclerotic plaques of CKD patients are more susceptible to rupture [7].

Many recent experimental studies addressed the pathogenetic mechanisms underlying arterial calcification in uraemia. Furthermore, clinical studies defined the extent and progression of aortic and coronary calcification. In addition to intimal calcification, calcification of the media is characteristic for CKD patients; its sequelae are increased

arterial stiffness resulting in increased cardiac afterload and increased CV mortality [8]. In CKD patients, apart from high phosphate and frequently also calcium concentrations, the main mechanism involved in vascular calcification is a deficiency of both local and systemic calcification inhibitors [1]. Vascular calcification is an active process with osteoblastic transdifferentiation of vascular smooth muscle cells (VSMC); the formation of hydroxyapatite resembles the process normally occurring in bone [9].

In the present study, we assessed the histology and the expression of candidate molecules potentially involved in the calcification of aortic intima and media of CKD patients compared with matched controls.

Material and methods

Patients

We included all consecutive non-diabetic patients with advanced CKD ($n = 31$) and selected controls without kidney disease matched for age and gender ($n = 31$) who had come to autopsy between January 2004 and July 2007 at the Department of Pathology of the University of Heidelberg. Ten patients had been on dialysis, and the others had died in end-stage renal failure. Information on CV risk factors (history of hypertension, nicotine abuse, diabetes etc.) and clinical evidence of CV disease was obtained from hospital records. Three 1 × 2 cm samples of abdominal aorta immediately distal to the renal arteries were excised and treated as described below.

Backscatter imaging and x-ray-analysis

X-ray analysis of the samples (Leo 440 Scanning electron microscope equipped with a Si–Li detector of Oxford Instruments GmbH, Wiesbaden Nordenstadt, Germany) was carried out at the Institute of Mineralogy, University of Heidelberg. The samples were enriched with uranium (U) for better discrimination of the vessel layers. Backscatter electron images of the sections (2 μm) were obtained before the analysis to find the optimal orientation for the sample profiles. After selecting the position for the measurements, two parallel line profile measurements were analysed using an analytical scan of 30 × 50 μm.

Major elements in the obtained spectra were carbon (C), uranium (U), oxygen (O), chloride (Cl), calcium (Ca) and phosphorous (P). The latter two were the elements of interest. The relative proportion of the elements in the investigated area field was quantitated and given in percent.

To avoid sampling errors, great care was taken to check whether corresponding anatomical structures were present in the consecutive sections used for the stains described below.

Immune histology and immune fluorescence

Additional specimens obtained from the same site of the aorta were fixed in 4% formalin, embedded in paraffin and examined immunohistochemically. Two investigators blinded with respect to the diagnosis used a semi-quantitative scoring system for the analysis (light microscopy; magnification ×200). The mean calculated concordance for the scores between the two investigators was between K = 0.75 and 0.82. As described previously [10], the intensity of staining was ranked on an arbitrary scale: grade 0 no staining; grade 1 faint staining; grade 2 positive staining involving up to 50% of the field of view; grade 3 positive staining involving more than 50%; grade 4 positive staining of all structures within the field of view.

Human-specific primary antibodies against core-binding factor alpha 1 (cbfa-1), osteocalcin (OC) (Abcam, UK), alpha smooth muscle actin (α-SMA) (Sigma-Aldrich, Germany), Pit-1 (Alpha Diagnostics, USA), Fetuin A (Genzyme, USA), matrix-gla-protein (MGP), osteopontin (OPN) (Immundiagnostik, Germany), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), bone morphogenetic protein 2 (BMP-2), tumour necrosis factor α (TNF-α), transforming growth factor beta 1 (TGF-β_1), phospho-p38, myeloperoxidase (MPO), Msx-2 (SantaCruz Biotechnologies, Germany), bone sialo-protein (BSP) (Chemicon, Germany), nitrotyrosine, superoxide dismutase (SOD)-Mn, SOD-Cu/Zn (Oxis International, USA), C-reactive protein (CRP) (Biotrend, Germany), CD-68 (Dako, Germany) were used. Appropriate biotinylated secondary antibodies [anti-rabbit (BioGenex, USA); anti-mouse or anti-goat (Jackson ImmunoResearch, USA)] were applied and were followed by the streptavidin-conjugated alkaline phosphatase (BioGenex, USA). For antigen visualization, Fast Red substrate (DAKO, Germany) was used. For immunofluorescence, secondary antibodies conjugated with Alexa fluor 488 (Chemicon, Germany) or Cy3 (Jackson ImmunoResearch, USA) were used. Negative controls were performed by omitting the primary antibody.

Statistical analysis

Data are given as mean ± SD (for normal distribution) or as median and range (for non-normal distribution). After testing for normal distribution, Student's *t*-test or the Mann–Whitney *U*-test was used as appropriate. The Kruskal–Wallis test or one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test, was used for multiple group comparisons, and *t*-test or Mann–Whitney test for comparisons between two groups. The correlation coefficient was calculated with the Spearman rank *R* correlation test. Multivariate analysis including age, gender, BMI, presence of hypertension, CV disease, diabetes and smoking status was performed for all parameters. The results were considered significant when the probability of error (P) was < 0.05.

Results

Patients

Age (CKD 68 ± 10 years versus controls 68 ± 11 years) and gender distribution (18 males and 13 females in either group) were comparable in patients with CKD and controls. A history of smoking was present in 26% of CKD patients and 23% of controls. Information on symptomatic CV disease tended to be more frequent in CKD patients compared with controls: hypertension 42% versus 23%; clinical signs of coronary heart disease 39% versus 19%; history of myocardial infarction 29% versus 10%; history of stroke 13% versus 6%.

Backscatter and x-ray analysis

In the aortic intima and in media of CKD patients, the % area exhibiting calcium/phosphorous deposits by x-ray analysis was significantly higher compared to controls. The same was true for calcium/phosphate content quantitated per unit volume (Figure 1).

Some markers of osteoblastic phenotype are upregulated in VSMC even without overt calcification in CKD

Staining for cbfa-1 was significantly more intense in calcified versus non-calcified specimens of the intima and media, but there was no difference between CKD and controls (Table 1).

A similar pattern of staining for Msx-2 was observed in the intima (Table 1). In contrast, in the media staining for Msx-2 was higher also in non-calcified specimens of CKD patients compared with controls (Figure 2a–d). The staining for Msx-2 in CKD was less intense, but broader compared with controls.

There was no detectable loss of staining for α-SMA in intima and media of calcified versus non-calcified regions and in CKD versus controls (Table 1).

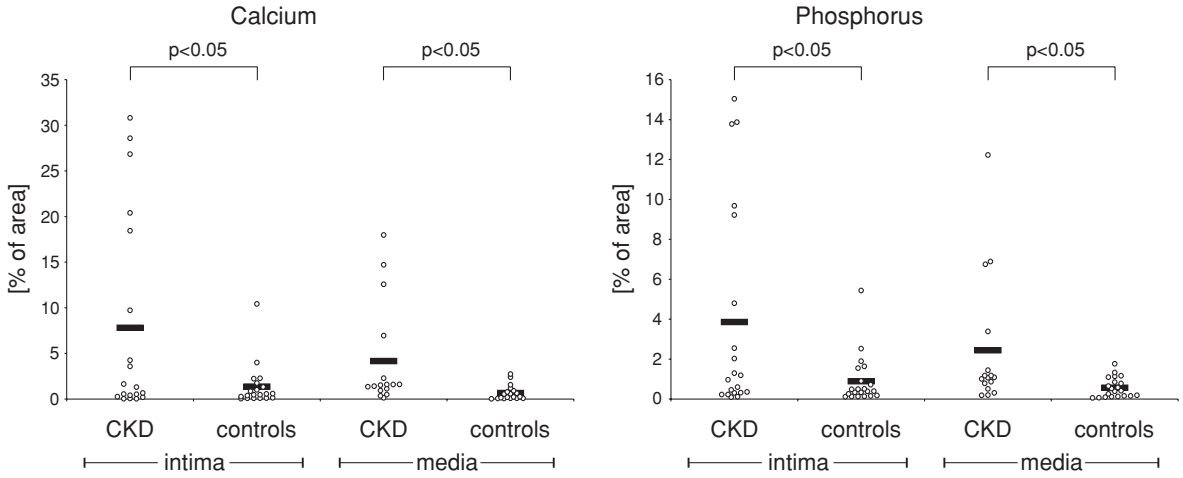

Fig. 1. Area of calcium and phosphorous deposition in the intima and media of the abdominal aorta of CKD patients and matched controls.

Staining for Pit-1 [type III Na^+-dependent phosphate (NaPi) transporter] was significantly more marked in the intima of calcified compared with non-calcified aorta specimens without any difference between CKD and controls (Table 1, Figure 2e–h). In contrast, a difference of the intensity of staining for Pit-1 was found in the media of calcified aorta specimens: staining was significantly more intense in CKD than in controls.

Staining for BSP was significantly more marked in calcified compared with non-calcified aortic specimens. In the intima, it was significantly more marked in non-calcified specimens from CKD patients compared with controls. In calcified media, staining for BSP was significantly more intense in CKD patients compared with controls (Table 1).

Staining for OPG and RANKL was not different between calcified and non-calcified aorta specimens and there was also no difference between CKD and controls.

Staining for RANK was significantly more marked in calcified compared with non-calcified aortic specimens. There was no difference between CKD and controls.

Staining for OC in the intima and media was significantly more marked in calcified compared with non-calcified aortic specimens from CKD patients. This difference was not observed in controls. In calcified aortic specimens, the staining for OC was significantly more intense in CKD patients compared with controls.

Staining for OPN was significantly more intense in calcified versus non-calcified aortic specimens (Table 1). Staining for OPN in non-calcified media was significantly more marked in CKD patients compared with controls.

Staining for BMP-2 was significantly more marked in both intima and media from CKD patients compared with controls (Table 1, Figure 2i–l). This was true both in calcified and non-calcified specimens.

Less Fetuin A is deposited in the aorta of CKD

Staining for Fetuin A was more marked in calcified versus non-calcified aorta specimens and particularly so in the area around calcifications. There was no difference of staining between CKD patients and controls in calcified aortic specimens, but a tendency of less staining in CKD patients was noted in non-calcified areas (Table 1).

Staining for MGP was more marked in calcified versus non-calcified aorta specimens. There was no difference between CKD patients and controls (Table 1).

Proteins involved in wall remodelling

Staining for (TGF-β_1) was significantly less marked in the intima of non-calcified aorta of CKD patients compared with controls (Table 1). No difference in staining for TGF-β_1 was observed in the media between calcified and non-calcified aorta specimens. There was also no difference between CKD patients and controls.

Staining for *phosphorylated p38* MAP kinase was significantly more marked in calcified compared with non-calcified aorta specimens (Table 1). There was no difference between CKD patients and controls.

Staining for transglutaminase 2 (TG2) in the intima was significantly more marked in calcified compared with non-calcified aorta specimens of controls, but not of CKD patients (Table 1, Figure 2m–p). In the media, staining for TG2 was significantly more marked in calcified compared with non-calcified specimens. There was no difference between CKD patients and controls.

Local markers of inflammation are increased in CKD

Staining for CRP was not different in intima and media of calcified versus non-calcified regions and also not between CKD patients and controls (Table 1).

In the intima, there were significantly ($P = 0.003$) more CD-68 positive macrophages present in calcified aorta specimens from CKD patients (20.9 ± 16.4 cells/mm^2) and controls (20.9 ± 13.1) compared to non-calcified aorta specimens from either CKD patients (6.4 ± 5.3) or controls (5.5 ± 3.4). In the media, there was no difference in the number of CD-68 positive macrophages between calcified and non-calcified regions, and there was also no difference between CKD patients and controls.

Table 1. Evaluation of the immunohistologic staining of the aorta (scores 0–4; three samples per staining per patient)

Parameter	CKD patients		Matched controls		ANOVA
	Non-calcified ($n = 8$ patients)	Calcified ($n = 23$ patients)	Non-calcified ($n = 16$ patients)	Calcified ($n = 15$ patients)	
Cbfa-1					
Intima	0.08 ± 0.17	1.57 ± 1.03*	0.28 ± 0.28†	1.36 ± 1.20*,‡	$P < 0.001$
Media	0.22 ± 0.54	0.92 ± 0.67*	0.19 ± 0.21†	1.10 ± 0.74*,‡	$P = 0.001$
Msx-2					
Intima	1.62 ± 1.37	2.98 ± 1.26*	1.54 ± 0.75†	2.55 ± 1.04*,‡	$P = 0.002$
Media	1.63 ± 0.91	1.78 ± 0.76	0.93 ± 0.58*,†	1.81 ± 0.90‡	$P = 0.008$
αSMA					
Intima	0.85 ± 0.65	1.49 ± 1.35	2.27 ± 1.54	2.16 ± 1.12	NS
Media	3.09 ± 0.51	3.15 ± 0.86	3.03 ± 1.02	3.52 ± 0.39	NS
Pit-1					
Intima	0.32 ± 0.56	1.88 ± 0.87*	0.85 ± 0.64†	2.02 ± 0.64*,‡	$P < 0.001$
Media	0.60 ± 0.77	2.29 ± 0.69*	0.92 ± 0.57†	1.51 ± 0.59*,†,‡	$P < 0.001$
Bone sialo-protein					
Intima	1.73 ± 0.66	1.87 ± 1.05	0.79 ± 0.87*,†	1.62 ± 0.99‡	$P = 0.010$
Media	0.39 ± 0.55	1.41 ± 1.09*	0.52 ± 0.66†	0.68 ± 0.67†	$P = 0.006$
Osteoprotegerin					
Intima	0.53 ± 0.74	0.43 ± 0.60	0.25 ± 0.47	0.38 ± 0.57	NS
Media	0.50 ± 0.30	0.39 ± 0.47	0.28 ± 0.36	0.22 ± 0.28	NS
RANKL					
Intima	0.61 ± 0.46	0.87 ± 0.83	0.71 ± 0.67	0.75 ± 0.82	NS
Media	0.62 ± 0.49	0.95 ± 0.96	0.59 ± 0.44	0.75 ± 0.86	NS
RANK					
Intima	0.43 ± 0.61	1.80 ± 1.22*	1.00 ± 0.60	1.21 ± 0.98	$P = 0.025$
Media	0.18 ± 0.27	1.49 ± 1.14*	0.69 ± 0.50†	1.02 ± 0.61*	$P = 0.006$
Osteocalcin					
Intima	0.59 ± 0.49	1.88 ± 0.79*	0.90 ± 1.09†	1.17 ± 0.75†	$P = 0.002$
Media	0.53 ± 0.52	1.26 ± 0.53*	0.51 ± 0.49†	0.82 ± 0.50†	$P = 0.001$
Osteopontin					
Intima	1.15 ± 1.01	2.11 ± 0.93*	0.37 ± 0.23†	1.91 ± 1.06*,‡	$P = 0.003$
Media	0.75 ± 0.49	1.18 ± 0.71	0.27 ± 0.39*,†	0.96 ± 0.43‡	$P = 0.005$
BMP-2					
Intima	1.81 ± 0.89	1.61 ± 0.80	0.37 ± 0.29*,†	0.44 ± 0.37*,†	$P = 0.007$
Media	1.47 ± 0.62	1.56 ± 0.62	0.38 ± 0.24*,†	0.48 ± 0.39*,†	$P < 0.001$
Fetuin A					
Intima	0.98 ± 0.82	1.72 ± 0.68	1.98 ± 1.12*	2.21 ± 1.22*	$P = 0.039$
Media	0.39 ± 0.33	1.43 ± 0.65*	0.96 ± 0.49*	1.51 ± 1.16*	$P = 0.015$
MGP					
Intima	0.37 ± 0.22	1.16 ± 0.61*	0.58 ± 0.70†	1.48 ± 0.69*,‡	$P = 0.002$
Media	1.05 ± 0.64	2.02 ± 0.69*	1.10 ± 0.72†	2.31 ± 0.54*,‡	$P < 0.001$
CRP					
Intima	1.85 ± 0.98	2.83 ± 1.37	2.31 ± 0.90	2.31 ± 0.76	NS
Media	1.10 ± 0.59	2.20 ± 1.33	1.76 ± 0.92	1.91 ± 0.81	NS
TNF-α					
Intima	1.50 ± 0.37	1.82 ± 0.87	0.75 ± 0.57*,†	1.39 ± 0.64‡	$P = 0.002$
Media	1.49 ± 0.53	1.22 ± 0.59	0.53 ± 0.41*,†	1.16 ± 0.56‡	$P = 0.001$
Nitrotyrosine					
Intima	1.94 ± 0.80	2.21 ± 0.84	0.63 ± 0.47*,†	1.13 ± 0.38*,†	$P < 0.001$
Media	1.76 ± 1.02	1.72 ± 0.80	0.44 ± 0.40*,†	0.93 ± 0.52*,†	$P < 0.001$
MPO					
Intima	1.31 ± 1.11	1.60 ± 0.98	0.83 ± 0.74	1.64 ± 1.32	NS
Media	0.55 ± 0.45	0.87 ± 0.73	0.44 ± 0.43	0.82 ± 0.75	NS
SOD-Mn					
Intima	1.78 ± 1.30	2.52 ± 1.06	1.21 ± 0.65†	1.99 ± 0.82	$P = 0.001$
Media	0.95 ± 0.64	1.54 ± 0.90	0.68 ± 0.40†	1.40 ± 0.85‡	$P = 0.005$
SOD-Cu/Zn					
Intima	1.66 ± 1.91	1.42 ± 0.92	1.33 ± 0.72	1.68 ± 1.12	NS
Media	1.80 ± 1.77	1.86 ± 1.23	1.61 ± 1.15	1.59 ± 0.89	NS
TGF-β1					
Intima	1.58 ± 1.37	2.79 ± 0.93*	2.29 ± 0.79*	2.91 ± 1.12*	$P = 0.024$
Media	1.02 ± 0.47	1.94 ± 0.97	1.38 ± 0.85	1.59 ± 0.86	NS
phospho-p38					
Intima	0.25 ± 0.19	1.22 ± 0.91*	0.45 ± 0.45†	1.33 ± 0.53*,‡	$P = 0.003$
Media	0.42 ± 0.29	1.81 ± 0.91*	0.59 ± 0.56†	2.14 ± 0.72*,‡	$P < 0.001$
Transglutaminase 2					
Intima	0.51 ± 0.48	0.52 ± 0.49	0.27 ± 0.22	1.22 ± 0.85*,†,‡	$P = 0.001$
Media	0.86 ± 1.30	1.23 ± 1.01	0.23 ± 0.21†	1.20 ± 1.13‡	$P = 0.015$

Significant differences versus *CKD patients non-calcified; †CKD patients calcified; ‡control cases non-calcified.

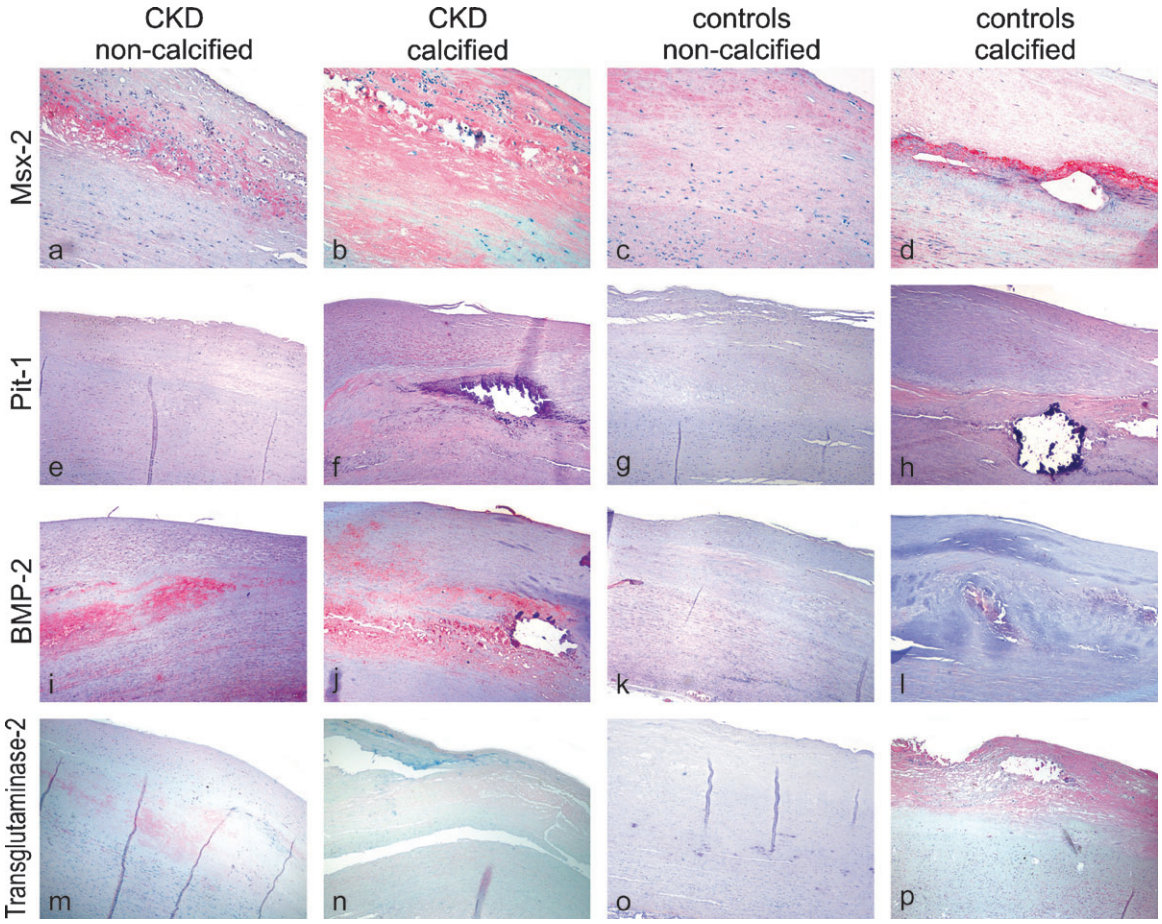

Fig. 2. Immune staining for Msx-2 (**a**–**d**), Pit-1 (**e**–**h**), BMP-2 (**i**–**l**) and transglutaminase-2 (**m**–**p**); representative images, magnification ×50.

Staining for TNF-α in the intima and media was significantly more intense in calcified versus non-calcified aorta specimens (Table 1). In non-calcified aorta specimens, the staining was significantly more marked in CKD patients compared with controls. In intima, the staining for TNF-α co-localized with CD-68 positive macrophages (Figure 3a–f). In media, the staining for TNF-α also co-localized with CD-68 in controls (Figure 3g–i) but was also seen independently of CD-68 in CKD patients (Figure 3j–l).

Oxidative stress is present in the aorta of CKD

Staining for *nitrotyrosine* was not different between calcified and non-calcified aorta specimens (Table 1). It was significantly more marked in CKD patients compared to controls both in the intima and media.

Staining for MPO was not different in the intima and media of calcified versus non-calcified regions and also not between CKD patients and controls (Table 1). Staining for MPO co-localized with CD-68 positive macrophages both in CKD patients and controls (Figure 3m–r).

Staining for the SOD-Mn isoform was significantly more marked in calcified compared with non-calcified aorta specimens (Table 1). There was no difference between CKD and controls.

Staining for the SOD-Cu/Zn isoform was not different between calcified and non-calcified aorta specimens, and there was also no difference between CKD and controls (Table 1).

Apoptosis is more marked in calcification and in CKD

A significantly higher number of tunel-positive cells was observed in calcified versus non-calcified aorta specimens in controls (Figure 4). This difference was not observed in CKD. Moreover, the number of apoptotic cells was lower in calcified specimens from CKD compared with controls.

Osteoblastic transdifferentiation correlates with TNF-α

The number of CD-68 positive macrophages did not correlate with the expression of MPO and TNF-α and only weakly correlated with the intensity of staining for nitrotyrosine ($r = 0.356$, $P = 0.049$) in media. The staining for BMP-2 significantly correlated with the staining for nitrotyrosine ($r = 0.519$, $P < 0.001$), TNF-α ($r = 0.403$, $P = 0.009$) and phosphorylated p38 MAP kinase ($r = 0.453$, $P = 0.006$), but not with the staining for cbfa-1. The staining for Msx-2 significantly correlated with the staining for BMP-2 ($r = 0.337$, $P = 0.025$), TNF-α ($r = 0.354$, $P = 0.037$),

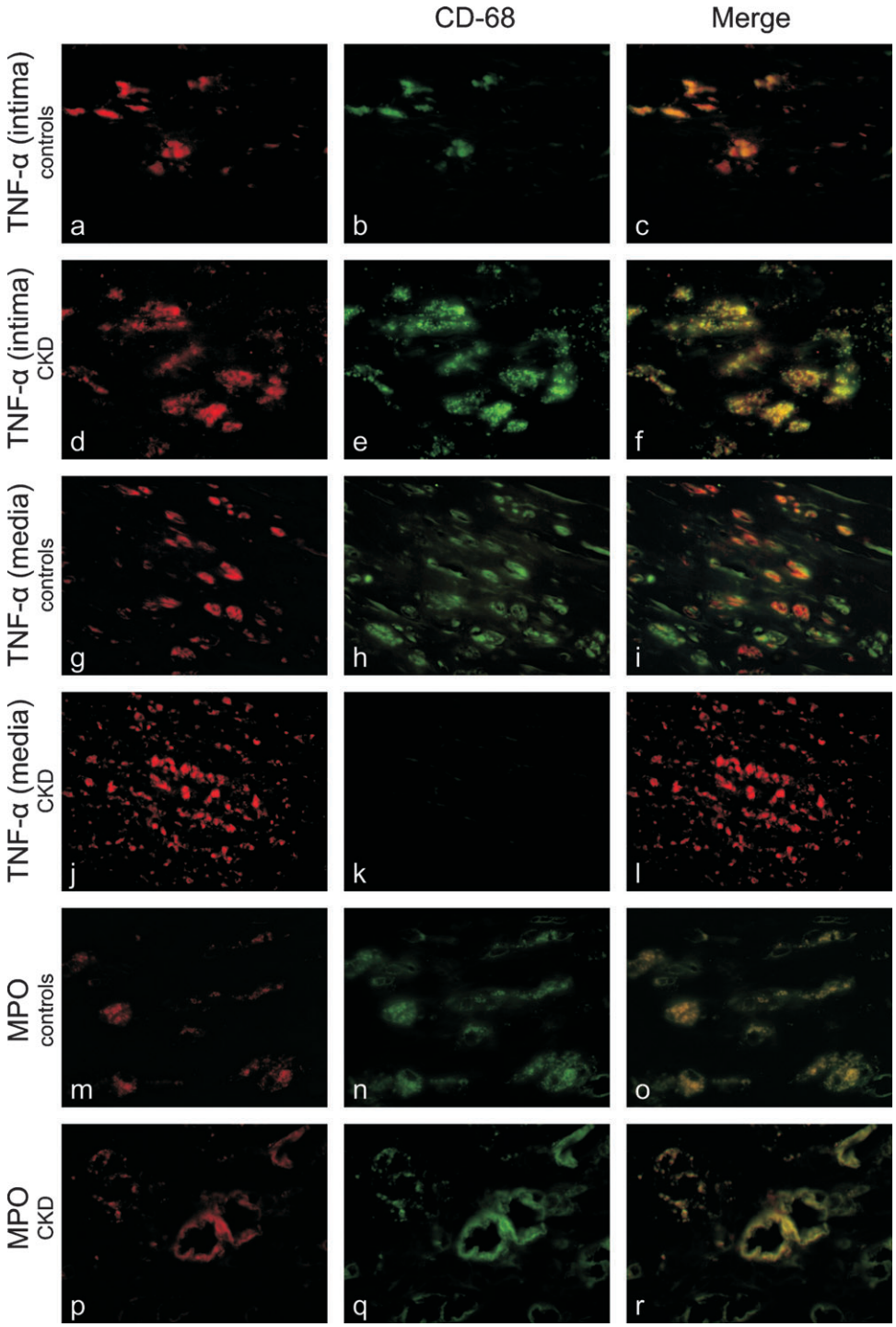

Fig. 3. Immune staining for CD-68 (green) and TNF-α or MPO, respectively (red), (representative images, magnification ×200). In the intima TNF-α co-localizes with CD-68 both of matched controls (**a**–**c**) and of CKD patients (**d**–**f**). In media TNF-α co-localizes with CD-68 in controls (**g**–**i**), but not in CKD patients (**j**–**l**). In contrast, MPO co-localizes with CD-68 in controls (**m**–**o**) as well as in CKD patients (**p**–**r**).

nitrotyrosine ($r = 0.431$, $P = 0.005$) and cbfa-1 (0.577, $P < 0.001$).

By multivariate analysis, none of the stainings in either intima or media were dependent on age, gender, BMI, presence of hypertension, CV disease, diabetes or smoking status (data not shown).

Discussion

In the present study, the calcified areas of the abdominal aorta were compared between CKD patients and matched controls. The salient findings are the more marked expression of Msx-2, BSP, BMP-2, TNF-α and nitrotyrosine in

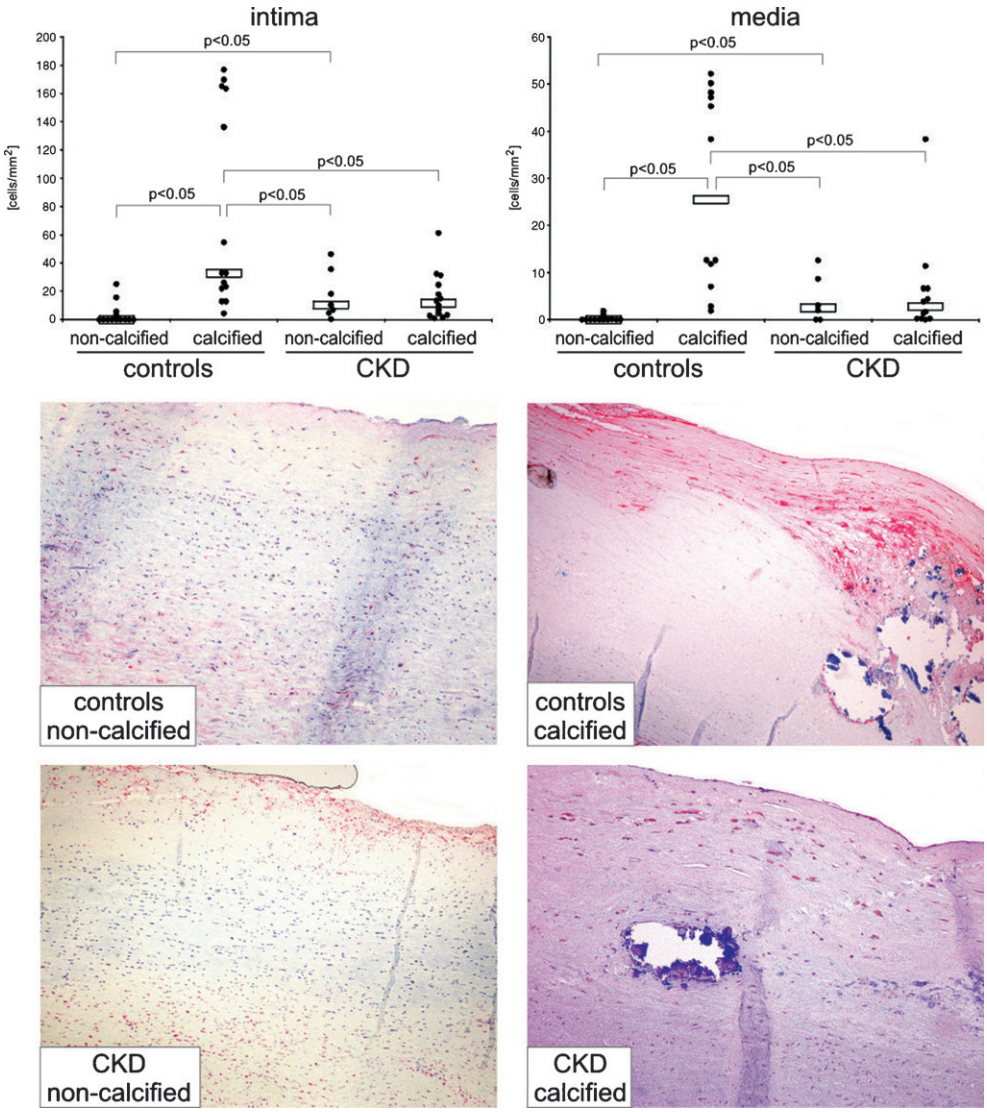

Fig. 4. TUNEL staining; assessment in intima and media and representative images, magnification ×50.

the aorta of CKD patients even in the absence of overt calcification. The very same proteins are markedly expressed in calcified aorta specimens of both CKD patients and controls. The increased staining for nitrotyrosine points to increased oxidative stress, and TNF-α to a pro-inflammatory milieu, both predisposing to calcification.

Using more sophisticated techniques, the present study confirms previous reports [8,11] that the calcium and phosphorus content of the aortic intima and media is higher in CKD patients compared with age- and gender-matched controls. The x-ray analysis confirmed that the mineral was deposited as hydroxyapatite.

We found a massive increase of apoptosis in calcified aortas from non-CKD patients. In aortas of CKD patients, apoptosis was increased even in the absence of calcification, but in contrast to the observation by Shroff *et al*. [11] no further increase was observed in calcified aorta specimens. Compared to the study of Shroff [11], we investigated the aorta, a central vessel, and not peripheral arteries. Furthermore, we examined calcification also in pre-dialysis patients. In our material, apoptosis seems to be a major contributor to calcification in non-CKD patients, but in CKD patients other factors apparently accelerate calcification beyond the effects of VSMCs apoptosis.

Osteoblastic differentiation is accompanied by upregulation of BMP-2 [12]. Of note, in our study, BMP-2 was upregulated in all CKD patients (independently of their calcification status), but not in matched controls. In patients without kidney disease, BMP-2 was also not related to plaque calcification [13]. Fukui *et al*. showed that TNF-α upregulates BMP-2 in human chondrocytes *in vitro* [14]. In line with that result, we documented a parallel increase of TNF-α and BMP-2 expression in human aorta of CKD patients. Moreover, in the study of Fukui *et al*., the TNF-α-dependent regulation of BMP-2 was mediated via p38 MAP kinase [14]. It is therefore of interest that we found an increase in phosphorylated p38 in parallel with the upregulation of TNF-α and BMP-2. Although the number of infiltrating macrophages per unit area was higher in calcified plaques compared to non-calcified

areas, such difference was not found in the media. In our study of the abdominal aorta of CKD patients, staining for TNF-α was found not only in infiltrating macrophages, but also in CD-68 negative cells (presumably resident cells). In contrast to what had been observed in calcified coronary plaques [7], deposition of CRP was not more intensive in calcified segments compared to non-calcified areas of the abdominal aorta of CKD patients.

Even in the absence of calcification, we found upregulation of Msx-2 and in parallel expression of TNF-α and BMP-2 in the aorta of CKD patients. This observation is in line with the direct effect of TNF-α on Msx-2 expression documented in experimental studies [15].

A diffuse increase of nitrotyrosine staining throughout the aorta of CKD patients points to increased oxidative stress creating a milieu favouring calcification. In line with previous observations of stenotic aortic valves [16], we observed that the expression of Msx-2 was co-localized with calcium deposits, but in contrast to that study [16], we failed to observe downregulation of SOD or the presence of cbfa-1 in non-calcified segments of the aorta.

It is known from *in vitro* experiments that in VSMC, calcification is modulated by the type III sodium-dependent phosphate co-transporter Pit-1 that is expressed by VSMC [17]. The present study in humans demonstrates upregulation of Pit-1 specifically in areas of heavy calcification. In agreement with experimental data, upregulation was more pronounced in individuals with CKD with and without dialysis. Pit-1 is a marker of phenotypic transdifferentiation of VSMC into osteoblast-like cells. This phenotype is also characterized by intracellular growth of hydroxyapatite microparticles. Experimental studies documented that the increased expression of Pit-1 is accompanied by upregulation of cbfa-1 and OPN. The present postmortem study confirms that the same occurs in the aortic intima.

OPN is a phosphoprotein found in mineralized tissues and acting as an inhibitor of apatite crystal growth [18]. Normally, OPN is not detectable in the vessel wall, but it is detectable at sites of calcification [19]. In agreement with these previous observations, we showed abundance of OPN in calcified areas of the abdominal aorta, but in CKD patients beyond that even in non-calcified areas of the aorta. In view of its role as an inhibitor of hydroxyapatite crystal growth, the presence of OPN may reflect initial stages of calcification not yet captured by the methods used in the present study.

The RANK/RANKL/OPG system plays a crucial role in regulating calcification and resorption processes in bone [20]. The components of this system are also present in the vascular wall; their role in vascular calcification is not fully understood [21]. Calcification of the aortic media is part of the phenotype of $OPG^{-/-}$ mice [22]. Some data in humans showed downregulation of OPG in calcified arteries [23], while others documented co-localization of OPG and calcium deposits [24]. Our findings argue against an important role in arterial calcification of CKD patients. All components of the system were detectable in the aorta of CKD patients, but the expression was not related to the calcification status, and the expression of the components of the RANK/RANKL/OPG system was not altered in CKD patients compared with controls without kidney disease. Other proteins relevant for vascular calcification include the protective components MGP and Fetuin A. These proteins were increased in calcified aortas, but there was no difference between CKD patients and controls.

TG2 stabilizes collagen and is thought to protect against plaque rupture [25,26]. It is of note that in our material, intimal expression of TG2 was increased in calcified areas of the aorta of controls without kidney disease, but not in CKD patients. This finding would be compatible with the known reduced plaque stability in CKD patients.

In summary, the expression of mediators of calcification is similar in the calcified abdominal aorta of CKD and non-CKD patients. In CKD patients, even in areas of the aorta without calcium deposits, markers of osteoblastic phenotype as well as indicators of oxidative stress and microinflammation, i.e. known promotors of calcification, are detectable. This presumably creates a milieu favourable to calcification. Local upregulation of TNF-α is accompanied by expression of Msx-2 and BMP-2 that are known promotors of osteoblastic transdifferentiation of VSMC and increased activation of the p38 MAP kinase.

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