Follow-up of arterial calcifications in rats with chronic renal failure: a quantitative *in vivo* and *in vitro* microtomography analysis

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Introduction

Vascular calcification (VC) is a prominent feature of cardiovascular disease in patients with chronic renal failure (CRF) [1]. We previously demonstrated that VC in rats with adenine-induced CRF can be detected and visualized by *in vivo* X-ray microtomography (micro-CT) [2]. In the present report, we used this method to assess the onset and evolution of the calcification process. This study also evaluated the analytical performance of micro-CT in the detection and quantification of the amount of calcified tissue by comparing micro-CT data to results from histology and atomic absorption spectrometry (AAS).

Methods

Male Wistar rats (n=108) were divided in different experimental groups to be studied at given time points. To induce CRF the rats were fed two different diets (adenine [1] and adenine + high protein [2]) for 4 weeks. VC was assessed by weekly scans of the living animals. The results of the *in vivo* image analysis were compared with *in vitro* high-resolution micro-CT (Skyscan 1076 and 1072, Kontich, Belgium), with histological data (Von Kossa staining and scoring) and with bulk calcium content measured by atomic absorption spectrometry (AAS).

Results

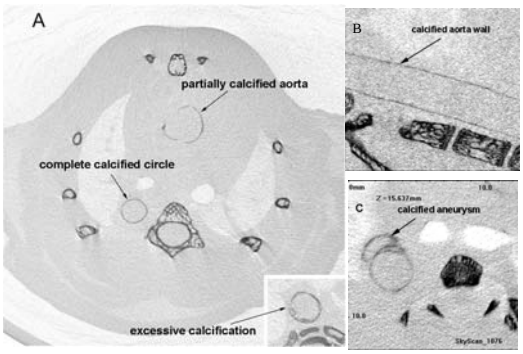

Figure 1: ***Aortic calcifications visualized by in vivo micro-CT*** (adenine model). A: cross-section with different degrees of calcification, B: longitudinal section, C: aneurysm; dark colours represent bone and calcified tissue (voxel size: 35*35*35µm).

	Post mortem techniques			X-ray microtomography				
Animal #	AAS wet tissue (mg/g)	Von Kossa (area %)	Histology scoring	Visual µ-CT (in-vivo)	Visual µ-CT (ex-vivo)	Volume of calcified tissue, µ-CT, mm³	Ca, µ-CT, (µg)	Ca density µ-CT (mg/cm³)
1	31.38	21.33	+	++	n.a.	n.a.	n.a.	n.a.
2	28.96	26.39	+	+++	n.a.	n.a.	n.a.	n.a.
3	26.28	10.4	+	+	+	5.989	330	55.3
4	22.38	22.65	+	+++	+	30.321	1990	65.8
5	19.98	20.35	+	++	n.a.	n.a.	n.a.	n.a.
6	19.26	22.95	+	++	n.a.	n.a.	n.a.	n.a.
7	16.94	17.83	+	++	+	27.479	1520	55.3
8	13.02	9.334	+	++	+	16.121	650	40.6
9	11.49	3.896	+	-	+	0.784	23	29.05
10	10.66	17.98	+	++	n.a.	n.a.	n.a.	n.a.
11	8.55	13.25	+	+	+	8.999	350	39.2
12	8.15	10.24	+	+	+	11.442	400	35.35
13	6.15	10.01	+	+	+	3.177	65	20.65
14	5.46	1.313	+	+	+	1.276	50	39.9
15	4.55	5.14	+	+	+	1.752	61	34.65
16	4.16	1.107	+	-	-	0.019	0.35	18.55
17	3.68	1.767	+	-	+	0.137	2.6	19.25
18	3.11	4.552	+	-	+	0.174	3.6	21
19	2.62	1.404	+	-	+	0.025	0.45	18.55
20	2.04	0.512	+	-	+	0.093	2.6	28.35
21	1.57	0.012	-	-	-	0.018	0.21	12.6
22	1.4	2.479	+	-	+	0.060	1	17.15
23	0.98	0.003	-	-	-	0.005	0.035	6.65
24	0.8	0.08	-	-	-	0.003	0.01	3.15
25	0.76	0.011	-	-	-	0.010	0.14	14
26	0.37	0.002	-	-	-	0.006	0.07	8.4
27	0.33	0.012	-	-	-	0.001	0.06	6.3
28	0.31	0.002	-	-	-	0.014	0.175	12.25
29	0.26	0	-	-	-	0.006	0.035	5.95
30	0.23	0	-	-	-	0.001	0.00035	0.35
31	0.19	0.002	-	-	-	0.020	0.315	15.05

Table 1: ***Comparison of in vivo and ex vivo micro-CT with AAS and histomophometric analysis*** (Von Kossa-staining). Rats (adenine diet) are sorted by the decrement of Ca content measured by AAS. NA=not analyzed. Scoring system for *in vivo* visual micro-CT : - negative, +: focal calcification or partially circumferential dense delineation of the aortic wall in at least two cross sections, ++: circumferential calcification of the aortic wall, +++: excessive circumferential calcification with regional increases in wall thickness. Volume, amount of calcium and calcium density were calculated from *ex vivo* micro-CT data.

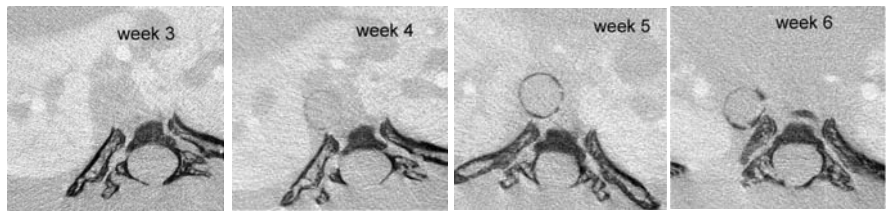

Figure 2: ***In vivo longitudinal follow-up by micro-CT of the development of aortic calcifications*** (adenine + low protein diet) (voxel size: 35*35*35µm). VC started focally, was initially seen histologically after 3 weeks and could be detected by *in vivo* micro-CT after 4 weeks of CRF. In several animals, density of the ectopic calcifications (at week 8) became comparable to that of bone.

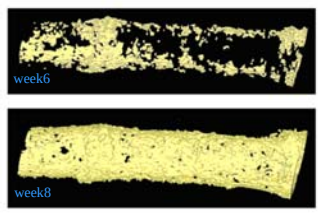

Figure 3: ***3D reconstruction of aortic calcifications*** (adenine +low protein diet) after *in vivo* scanning. Volume of caclified tissue at week 6 was 1.14mm^3, at week 8 it reached 7.77mm^3 (+582%) .

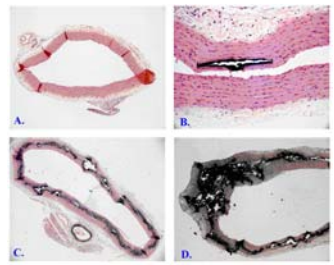

Figure 4: ***Histological images of different degrees of calcification*** (von Kossa staining). A: Aorta cross-section without calcification (0%), B: calcified spot (1-3%), C: completely circumferential calcification (~10%), D: excessive calcification (~20%). These results validate the observations shown in figure 1.

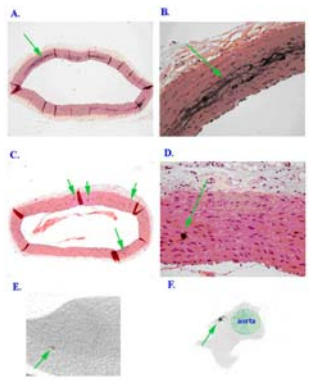

Figure 5: ***Difference in detection limits between histology, AAS and micro-CT***. A: "sand-type" Von Kossa positivity. Each grain is too small to be detected by micro-CT,
B: magnified image of the affected area with a lot of tiny calcifications, C: small calcified spot that is detected histomorphometrically, but can be considered as statistical noise by micro-CT, D: magnified image of individual spot, E: micro-CT detects a single, focal Ca deposit that brings very small contribution to AAS signal and can be easily missed in histology due to sampling bias, F: micro-CT image showing dense focus outside the aorta wall which contributes to the micro-CT-based Ca measurement when situated inside the selected region of interest, but will not be taken into account in histomorphometry.

Conclusions

As confirmed by traditional destructive methods, micro-CT proved to be effective for the follow-up of aortic calcifications at all different time points. "Positive" animals, with ectopic calcifications higher than 5 mg/g tissue, could be distinguished from living rats that did not calcify. Sensitivity of *in vivo* micro-CT was high enough to detect calcifications in 70% of the animals. After sacrifice, *in vitro* micro-CT analysis accurately quantified aortic calcifications (with almost 95% reliability), providing a complete analysis of both degree and distribution of the calcification.
Therefore, micro-CT is a promising non-invasive imaging technique allowing quantification of ectopic calcification both in living animals and in extracted tissues.

[1] Goodman, W. et al. (2000). *N Engl J Med.* **342**, 1478-1483
[2] Persy, V. et al. *Arteriosclerosis Thrombosis and Vascular Biology*, 2006, **26** : 2110.
[3] Price, PA. et al. (2006) *Kidney Int.* **70**, 1577-1583

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