ESRF	**Experiment title:** The Onset and Development of Mineral Deposition in the Pathophysiolgy of Vascular Calsification. An Xray Diffraction Study at Nano Meter Scale.	**Experiment number**: MD-292
Beamline: ID-13	**Date of experiment**: from: 12/11/2007 to: 19/11/2007	**Date of report**: 30/06/2008
Shifts: 12	**Local contact(s)**: Manfred Burghammer	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Steven VERBERCKMOES*(1), Geert BEHETS(1)*, Patrick D'HAESE(1), Dagmar-Christiane FISCHER(2)*, Dieter HAFFNER(2), Susanne JUNG*(3), Dominik MULLER(3), Ellen NEVEN(1), Veerle PERSY(1), Uwe QUERFELD(3)

(1) Dept. of Pathophysiology, University of Antwerp, Antwerp, Belgium

(2) AG Experimentelle Nephrologie Universitäts-Kinder- und Jugendklinik, Rostock, Germany

(3) Clinic of Pediatric Nephrology, Charité – Campus Virchow Clinic, Berlin, Germany

Report:

It has been estimated that 4.5 Million Europeans suffer from renal disorders. Although the elderly are disproportionally affected, renal disease is also a condition that severely affects children. In these patients, the frequent occurrence of vascular disease is often accompanied by arterial calcifications (Foley, Parfrey, and Sarnak). In recent years, the insight has emerged that ectopic calcification is a tightly regulated process, and results from an imbalance between inhibitors (such as fetuin and matrix Gla protein) and inducers of mineralisation (such as elevated serum calcium-phosphate product) (Ketteler, Gross, and Ritz;Moe et al.). A transdifferentiation of vascular smooth muscle cells towards cells with an osteogenic phenotype, expressing the major bone specific proteins, is a key event in the process of media calcification. Based on these observations it is generally assumed that the mineral deposited in the vascular wall has the physicochemical properties of hydroxyapatite, the main mineral compound of bone. Until now, however only very limited physicochemical evidence hereto has been reported in literature.

By applying µ-X-ray diffraction in the setting of a previous experiment (SC-1605) performed at the ESRF beamline ID22/ID18F, we were able to characterize the mineral phase deposited in two frequently used animal models of uremia-related vascular calcification, i.e. adenine-treated rats exposed to a high phosphate diet and remnant kidney rat treated with vitamin D. As more or less expected, in the adenine-treated model micro-crystalline apatite was found as the major mineral phase. The high resolution of synchrotron based µ-diffraction further allowed us to demonstrate at the ultra-structural level, that central calcified apatitic regions were accompanied by deposition of amorphous calcium-phosphate, at the outer edge of the calcified deposits allowing us to hypothesize this to be an early calcium-phosphate precipitate which matures over time to apatite.

These novel findings shed new light on the physicochemistry of vascular calcification, a research area that has been ignored for a long time. However, questions about the onset, temporal evolution and reversibility of the mineral deposition in this process remained unsolved. Especially the investigation of the maturation process from amorphous calcium phosphate to micro-crystalline apatite deserves more attention. In this project, which is a continuation project of MD-285@ID18F, we aimed to characterize the time-dependency of the mineral phase at a nanometer scale in aorta samples of rats in which chronic renal failure and subsequent vascular calcification was induced by the application of an adenine-rich/low-protein diet. In these experiments rats were sacrificed at different time points, covering the

different steps in the development of vascular calcification (Figure 1). We aimed to investigate the same samples as in the previous experiment MD-285 to obtain complementary data at a higher resolution.

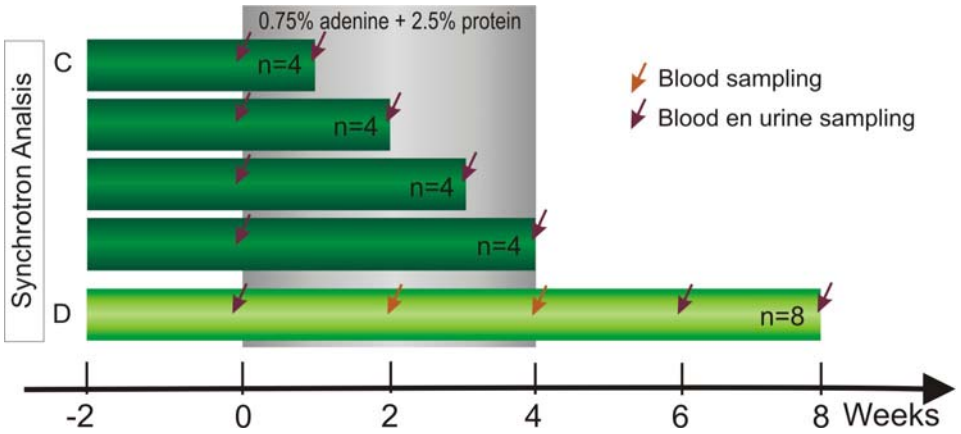

Figure 1: Set up of the experiment

Like mentioned in the experimental report of proposal MD-285 the obtained tissue samples of the large blood vessels were fixed and embedded in paraffin before the staining of the calcium deposits by Van Kossa staining (Figure 2). By the application of a semi-quantitative scoring system the appearance of the calcified zones in the aorta and their time-dependency were scored (score 0: no calcifications; score 1: focal calcifications only; score 2: partial circumferential calcified zones and score 3: cirumferential calcifications; Figure 3). Based on the results of the scoring of the calcified zones, only samples with positive Von Kossa staining were selected for further analysis by synchrotron radiation analysis.

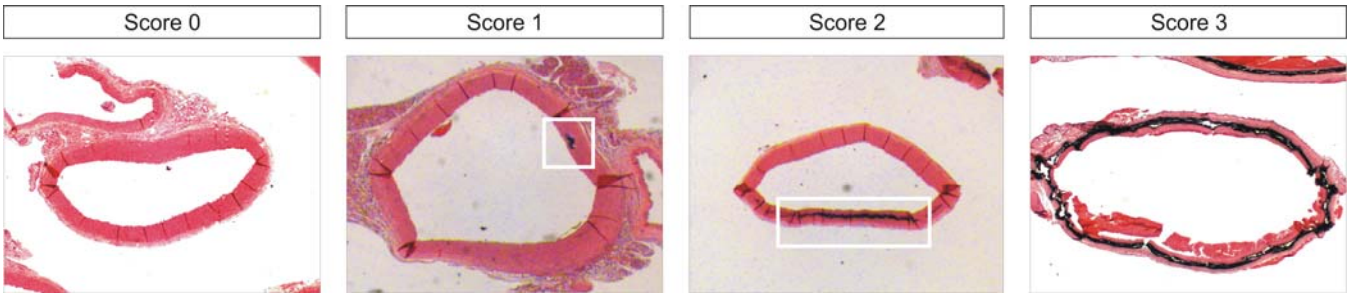

Figure 2: Illustration of the various degrees of vascular calcification and the application of the semi-quantitative scoring system: score 0: no calcifications; score 1: focal calcifications only; score 2: partial circumferential calcified zones and score 3: cirumferential calcifications.

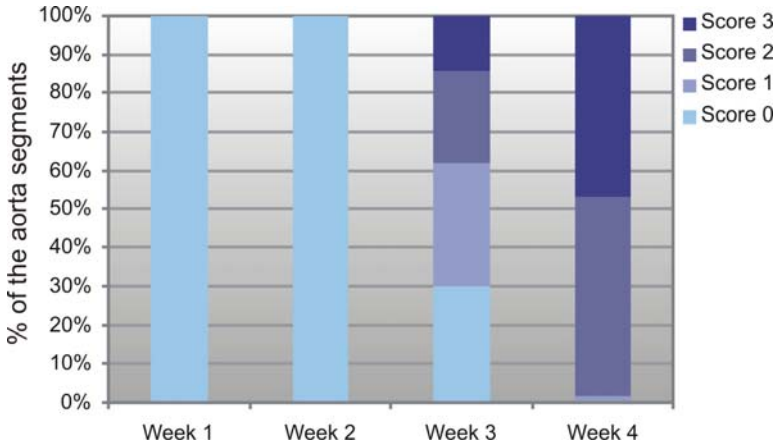

Figure 3: Time-course of the development of vascular calcification in the rat aorta visualized by the above mentioned scoring system. First calcifciations can be observed from week 3 on. Based on these results samples were chosen to be analysed by sychrotron techniques.

On the Von Kossa stained section a region of interest of a calcified zone was identified and subsequentially indicated on the sequential unstained analytical tissue section which was analyzed by synchrotron µ-fluorescence at beamline ID-18F with a 12.9 keV beam with a focal spot size of 1 x 1 µm, a resolution which was about 20 times higher than in experiment MD-285. In this way complementary data to those obtained during MD-285 were recorded. A Ca-mapping of the calcified

region of interest was constructed on which several line scans for combined fluorescence and diffraction analysis were performed. During these line scans diffraction patterns were recorded for every step of the scan. After integration of the diffraction rings the obtained diffractograms were used to identify the mineral phase and to estimate the variation in crystallinity through the sample.

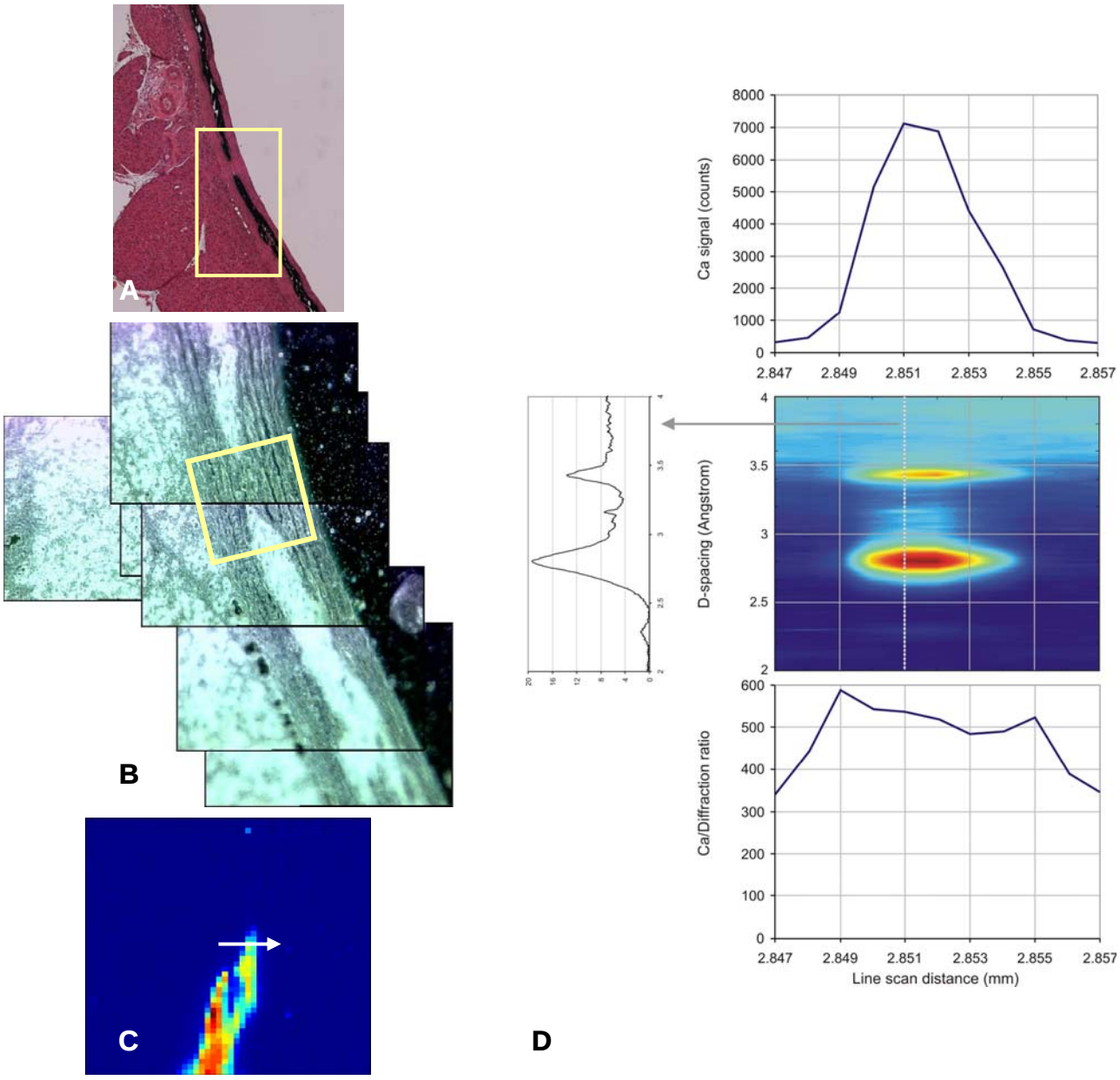

Figure 4: Typical example of a calcified zone investigated by µ-fluorescence and diffraction analysis with panel A: Von Kossa positive region of the investigated sample, panel B: Unstained sequential tissue section on which the investigated region is indicated, panel C: Ca-fluorescence mapping of the investigated calcified region, the arrow indicated the path of the line scan represented in panel D. On the upper figure of panel D the Ca-fluorescence profile of the line scan is represented. The colored panel represents the evolution of the diffraction pattern through the line scan. In the lower panel the Ca/diffraction is shown which shows a higher ratio at the edges of the calcified region. The latter profile could be indicative for the presence of an amorphous calcium phosphate precipitate at the beginning and the end of the line scan, however further investigations are needed to confirm this hypothesis.

In all samples the mineral phase could be identified as poorly crystalline apatite with an amorphous precursor phase at the edges of the calcified zone (Figure 4). More detailed investigations to confirm the presence of such an amorhous phase such as scanning electron microscopy to asses Ca/P ratios or Raman spectroscopy are needed. This will also contribute to further interprete the importance of the amorphous phase at this specific ultrastructural location.

Reference List

Foley, R. N., P. S. Parfrey, and M. J. Sarnak. "Epidemiology of cardiovascular disease in chronic renal disease." J Am Soc.Nephrol. 9.12 Suppl (1998): S16-S23.

Ketteler, M., M. L. Gross, and E. Ritz. "Calcification and cardiovascular problems in renal failure." Kidney Int.Suppl.94 (2005): S120-S127.

Moe, S. M., et al. "Role of calcification inhibitors in the pathogenesis of vascular calcification in chronic kidney disease (CKD)." Kidney Int. 67.6 (2005): 2295-304.