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Medial artery calcification of uremic patients: a histological, histochemical and ultrastructural study

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Summary. Recent findings suggest that vascular calcification (VC) is an active process similar to bone mineralization, the vascular smooth muscle cells (VSMCs) undergoing phenotypic differentiation into osteoblastic cells and synthesizing calcification-regulating proteins found in bone. This study has investigated the VC process of uremic patients, with a morphologic approach. Epigastric artery samples from 49 uremic, non-diabetic patients were taken during kidney transplantation. Sections from paraffin-embedded samples were stained with hematoxylin/eosin and von Kossa. CD68 was immunohistochemically detected, and sections from frozen samples were stained with Oil Red O. Deeply calcified samples were stained with Picrosirius Red, PAS, and Alcian blue. Specimens from one patient with moderate and one with severe VC were examined under the electron microscope. None of the samples had atherosclerosis. Calcifications were found in the media of 38 patients. In 23, dot-like calcifications were irregularly scattered near the adventitia (light VC); in 11, granular calcifications formed concentric rings near the adventitia (moderate-advanced VC); in 4, zones of consolidated calcifications were found (severe VC). These zones were poor in collagen, glycoproteins and proteoglycans. In cases with moderate or severe VC, VSCMs showed necrotic changes. Matrix vesicles could be recognized in the extracellular spaces. In cases with severe VC, uncalcified or partially calcified membranous bodies were found, together with Liesegang rings. Patches of fibrin were also found. These findings point to a mainly degenerative mechanism of VC, which proceeds from the outer portion of the media. An active mechanism, however, cannot be excluded. A unifying hypothesis is suggested.

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Introduction

Vascular calcification is due to the deposition of calcium phosphate, most often as hydroxyapatite, in the vessel wall. Calcification may occur in the intima (atherosclerosis), often associated with aging, and in the media (Mönckeberg's sclerosis), as, for instance, in diabetes and uremia (Trion and van der Laarse, 2004). The differences between intimal and medial calcifications imply different etiologies, although both of them occur through the vascular smooth muscle cells (VSMCs) regulation. Arterial calcification has been conventionally viewed as a passive, degenerative process. Recent findings suggest, however, that vascular calcification is an organized, regulated process similar to mineralization in bone tissue (Moe and Chen, 2004; Shroff and Shanahan, 2007). In other words, the VSMCs, under certain stimuli, could undergo a phenotypic conversion into osteoblastic cells and to synthesize transcription factors (e.g., Runx2/Cbfa1 and Osterix) and many of the calcification-regulating proteins commonly found in bone (e.g., bone morphogenetic proteins, alkaline phosphatase, osteopontin, osteonectin, osteocalcin, matrix Gla protein). This hypothesis is also supported by the electron microscope finding of vesicular particles, identical to matrix vesicles, in the matrix of the atherosclerotic plaque of the intima (Bobryshev et al., 2007), as well as in the calcifying media of uremic patients (Moe and Chen, 2004). Matrix vesicles are extracellular membrane-bound structures that normally bud-off from the outer plasma membrane of the chondrocytes, osteoblasts and odontoblasts. They are frequently observed at sites of initial calcification and

are thought to serve as the nidus for mineral nucleation by concentrating calcium and phosphate (Bonucci, 2007).

Matrix vesicle shedding from VSMCs has been described in atherosclerotic lesions and can occur as a result of necrotic cell death (Vattikuti and Towler, 2004; Bobryshev et al., 2007). In the media of arteries of uremic patients the beginning of the calcification process has been attributed to extracellular vesicular structures, which originate from degenerated and necrotic VSMCs (Ejerblad et al., 1979). Recent *in vitro* studies, however, support the possibility that microvesicle generation is mainly associated with VSMC apoptotic death. VSMCs cultured from the medial layer of human aortas spontaneously form multicellular nodules in which, besides calcium crystals, structures similar to matrix vesicles can be identified (Proudfoot et al., 1998). It has been suggested that VSMC-derived apoptotic bodies have similarities with matrix vesicles, since they are able to concentrate and to crystallize calcium, and that, therefore, apoptosis might initiate vascular calcification (Proudfoot et al., 2000). In line with these results, the TUNEL method, a staining technique for fragmented DNA usually applied for the histological detection of apoptosis, was described to be positive, both in the intima of atherosclerotic lesions (Crisby et al., 1997) and in the media of dialysis children (Shroff et al., 2008).

Although assuming that vascular calcification has many similarities with bone formation, it is reasonable to admit that some differences exist between the pathological and the physiological ossification process (Bobryshev et al., 2007). Whether in the vessel wall matrix vesicle production is due to cell death by necrosis or apoptosis is not yet fully understood. Vascular calcifications are of frequent occurrence in patients with chronic renal failure both in conservative stage and in hemodialysis treatment. In these patients, extensive medial calcification causes increased arterial stiffness, which is mechanistically linked with systolic hypertension, left ventricular hypertrophy, and reduced coronary perfusion (Schoppet et al., 2008). The high incidence of cardiovascular disease, i.e. heart failure, sudden cardiac death, acute coronary syndrome, and peripheral arterial disease, is the leading cause of death in patients treated with dialysis (Moe and Chen, 2004), who undergo 30-fold higher mortality than the general population (Schoppet et al., 2008). The present morphological study has been carried out to further investigate the medial artery calcification process in uremic patients, with the principal aim of examining thoroughly the VSMCs and their death.

Materials and methods

Samples of inferior epigastric arteries were taken from 49 hemodialyzed patients undergoing renal transplantation. The patients consisted of 35 males and 14 females, having a mean age of 48.88±14.49 years and a mean dialytic age of 99.17±105.10 months. The mean clinical laboratory values were as follows: serum calcium 9.26±0.62 mg/dl (normal range 8.5-10.2); serum phosphorus 5.37±1.02 mg/dl (normal range 3.0-4.5); serum alkaline phosphatase 170.2±84.6 mU/ml (normal range 35-125); serum parathyroid hormone 201.7±126.8 pg/ml (normal range 10-65). None of the patients was affected by diabetes.

Following the procedure described by Moe et al. (2002, 2003), the proximal portion of the inferior epigastric artery was clamped and a vessel sample about 2-3 cm long was removed, during the kidney transplantation. Branches of renal artery were obtained from two kidney donors and used as controls. Immediately after being removed, all samples were placed in phosphate buffer solution (PBS) containing 0.1% DEPC (diethyl pyrocarbonate; Sigma-Aldrich, St Louis, MO, USA). Each vessel was then transversally cut into 4-6 segments, 3-5 mm long, using a single edge blade. Half specimens were fixed in 4% para-formaldehyde in 0.1 M phosphate buffer at pH 7.2 and then processed for paraffin embedding. The other half was immersed in OCT embedding compound (Tissue-Tek, Zoeterwoude, NL, EU), frozen by isopentane-liquid nitrogen, and stored at -80°C. Sections from paraffin blocks were stained with hematoxylin/eosin (HE) for routine examination. The von Kossa method for calcium phosphate was used to detect calcifications. VSMCs and macrophages were immunohistochemically detected using mouse monoclonal antibody to human α-SM-actin and to CD68 (M0851 and M0814, DakoCytomation, Glostrup, DK, EU), respectively. Sections from frozen samples were stained with Oil Red O for lipids. The morphological classification scheme proposed by Virmani et al. (2000) was adopted for detecting and graduating the atherosclerotic lesions, if any. In the media, the von Kossa-positive areas were semi-quantitatively scored as calcifications of light degree, when only a few dot-like, positive foci were present, of moderate degree, when calcifications formed a few almost complete rings in the artery wall, of advanced degree, when calcification rings were numerous involving, in some cases, the full-thickness of the media, and of severe degree when large and patchy areas of consolidated calcium deposits were present. To avoid as much as possible cutting tears in sections, in all patients with severe calcification at least one of the 2-3 paraffin-embedded specimens was de-paraffinized, decalcified in 10% EDTA in 0.1 M phosphate buffer at pH 7.0 and re-embedded in paraffin. Sections from these specimens were stained with HE, Picrosirius Red for collagen, periodic acid-Schiff (PAS) for glycoproteins, and Alcian blue at pH 2.5 for acid proteoglycans. One specimen from three patients with moderate calcification was de-paraffinized, post-fixed for 1 h at room temperature in 1% osmium tetroxide, dehydrated in ascending series of ethanol and embedded in Epon. Semi-thin sections, about 1 μm thick, were stained with azure II-methylene blue for light microscope examination; ultra-thin sections (80-100 nm thick) were stained with uranyl acetate and lead citrate and examined using a Philips CM10 transmission electron microscope (TEM). One

specimen from two patients with severe calcification was de-paraffinized and post-fixed for 1 h at room temperature in 1% osmium tetroxide. Specimens were then 'en bloc' stained for 1 h with 2% aqueous uranyl acetate, dehydrated in ascending series of ethanol and embedded in Epon. Semi-thin sections from these specimens were stained with azure II-methylene blue, while ultra-thin sections were stained with lead citrate.

Results

The light microscope examination did not show atherosclerotic lesions. Non-atherosclerotic intimal thickenings were found in twelve patients: in ten of them intimal thickening was due to accumulation of α-SM-actin+ VSMCs among which small, CD68+ macrophagic cells were occasionally present; in the other two patients, intimal xantoma or "fatty streak" were found, consisting in the focal accumulation of macrophagic foam, Oil Red O-positive cells. No necrotic core, fibrous cap or calcifications were recognizable. In 37 patients, the tunica intima was thin and contained a few VSMCs, as normal.

The von Kossa stained sections showed that calcifications were absent in 11 patients (Fig. 1A), who did not show differences with respect to the controls. In the other 38 samples, calcifications were confined to the tunica media. They were semi-quantitatively scored as light (no.=23), moderate to advanced (no.=11), and severe (no.=4). In lightly calcified samples, dot-like

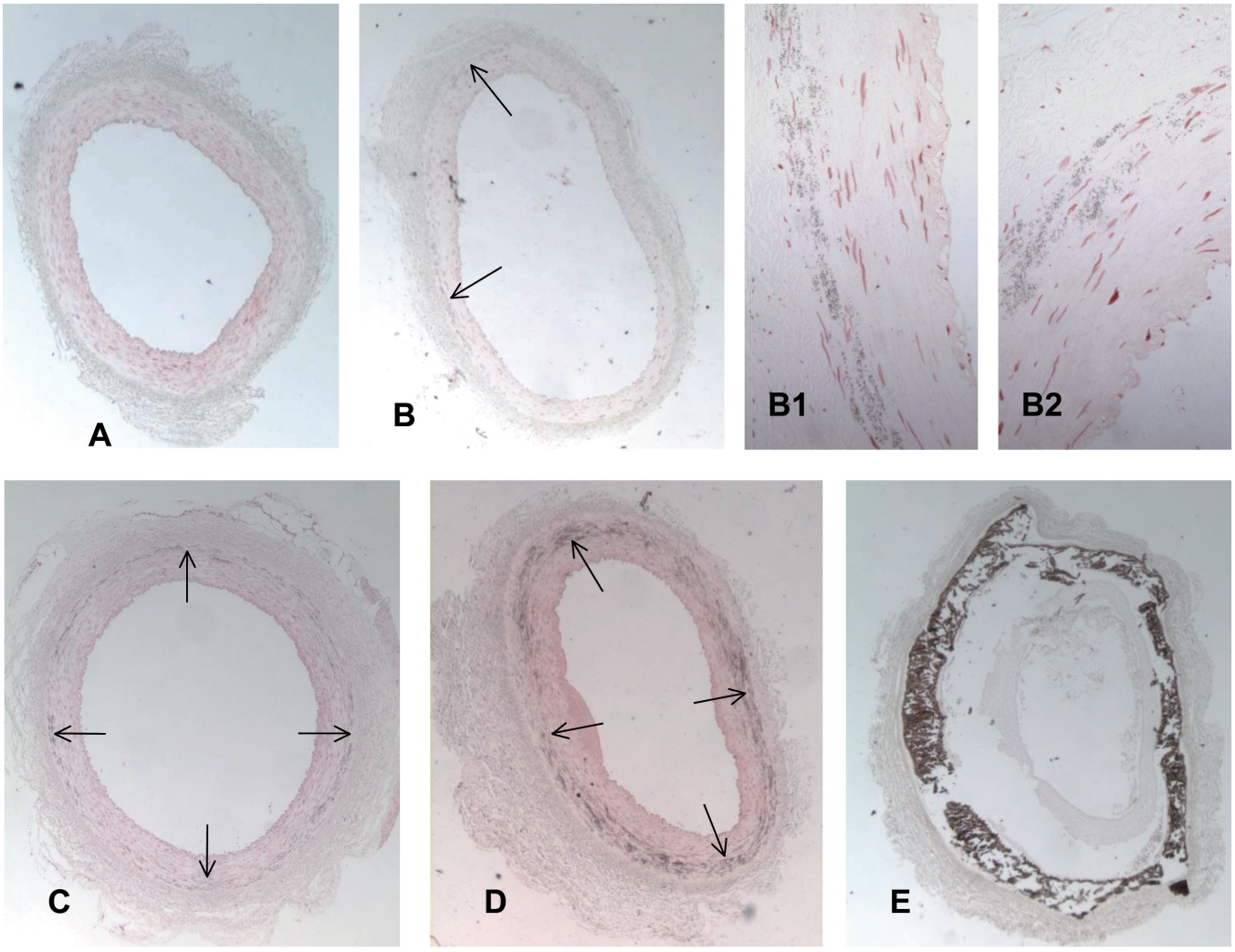

Fig. 1. Different microscopic pictures of the tunica media: **A)** sample without calcification; **B)** lightly calcified sample: two dot-like calcification foci near the adventitia (arrows); B1 and B2) higher magnifications of B, corresponding to the calcification spots on the bottom and on the top of B, respectively; **C**) moderately calcified sample: a ring of granular calcium deposits in proximity of the adventitia (arrows); **D)** sample with advanced degree of calcification: approximately concentrical, circumferential rings of calcium deposits involving most of the media (arrows); **E)** severely calcified sample: calcifications are consolidated and extend to full-thickness of the tunica media. Sections stained with von Kossa, counterstained with 1% Neutral Red; objective magnification A-E, x 2.5; B1, B2, x 20

calcification foci were scattered between VSMCs. They were typically more numerous in the outer third of the media, next to the adventitia (Fig. 1B,B1,B2). In the 11 patients with moderate to advanced calcifications, circumferential, almost complete rings of granular calcium deposits were found which involved variable portions of the media, but were more marked in proximity of the adventitia (Fig. 1C,D). In the 4 samples characterized by severe calcifications, large and patchy areas of consolidated calcium deposits were present; they were so hard that artefactual tears could not be avoided during cutting of paraffin embedded samples (Fig. 1E). Ossified areas, i.e., trabecular-like structures with abundant collagen fibrils and/or with osteocyte-like cells, were never found.

In semithin sections obtained from undecalcified,

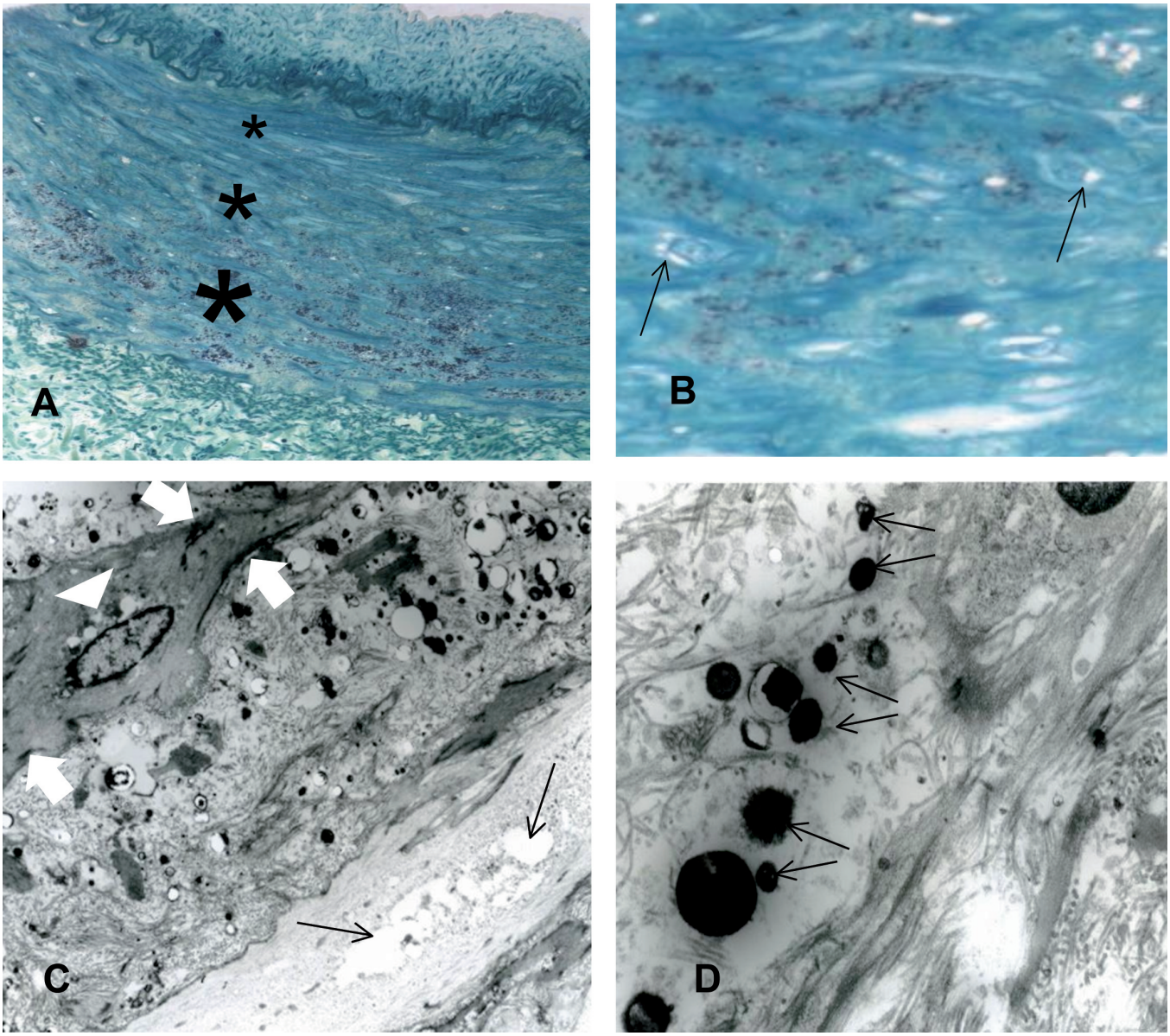

Fig. 2. Epon embedded, undecalcified samples with moderate medial calcifications: **A)** three zones are recognizable within the media: outer, with dot-like calcifications (large asterisk); intermediate, with abundant uncalcified extracellular matrix (medium asterisk); internal, with normal morphology (small asterisk); **B)** clear vacuoles in correspondence of some VSMSs (arrows); **C)** cytoplasmic vacuoles in proximity of the nucleus (arrowhead) of a cell (upper part of the figure) that contains myofilaments and dense bodies next to the plasmalemma (thick arrows) characteristic of contractile smooth muscle cells; the cell at the bottom contains very few myofilaments and large and numerous cytoplasmic vacuoles (arrows); **D)** some electron-dense bodies, morphologically similar to matrix vesicles, are present (arrows) in the extracellular matrix, mixed with sparse collagen fibrils. A and B: semithin sections stained with von Kossa and Azure II-Methylene Blue; objective magnification x 20 and x 40, respectively. C and D: ultrathin sections stained with uranyl acetate and lead citrate; electron micrograph x 7,375 and x 38,750, respectively.

Epon embedded samples with moderate calcifications, three zones could be distinguished within the tunica media: an external zone, near the adventitia, characterized by extracellular matrix with dot-like and granular calcifications; an intermediate zone, in which abundant uncalcified extracellular matrix was present between the VSMCs; an internal zone, adjacent to the intima, where the VSMCs were juxtaposed and the extracellular matrix was rather scarce (Fig. 2A). At higher magnification, clear cytoplasmic vacuoles could be found in several VSMCs, particularly those located in the external and intermediate zones (Fig. 2B). These vacuoles were more clearly visible under the TEM: a few small cytoplasmic vacuoles were visible near the nucleus of VSMCs that showed the characteristic myofilaments and dense bodies. A few of these cells, however, were almost devoid of myofilaments and the cytoplasm contained large and numerous vacuoles (Fig. 2C). In the extracellular matrix, sparse collagen fibrils, and several roundish electron-dense bodies, morphologically similar to matrix vesicles, could be identified (Fig. 2D).

Severe calcified specimens, which could not be cut without tears (Fig. 3A) because of their hardness and,

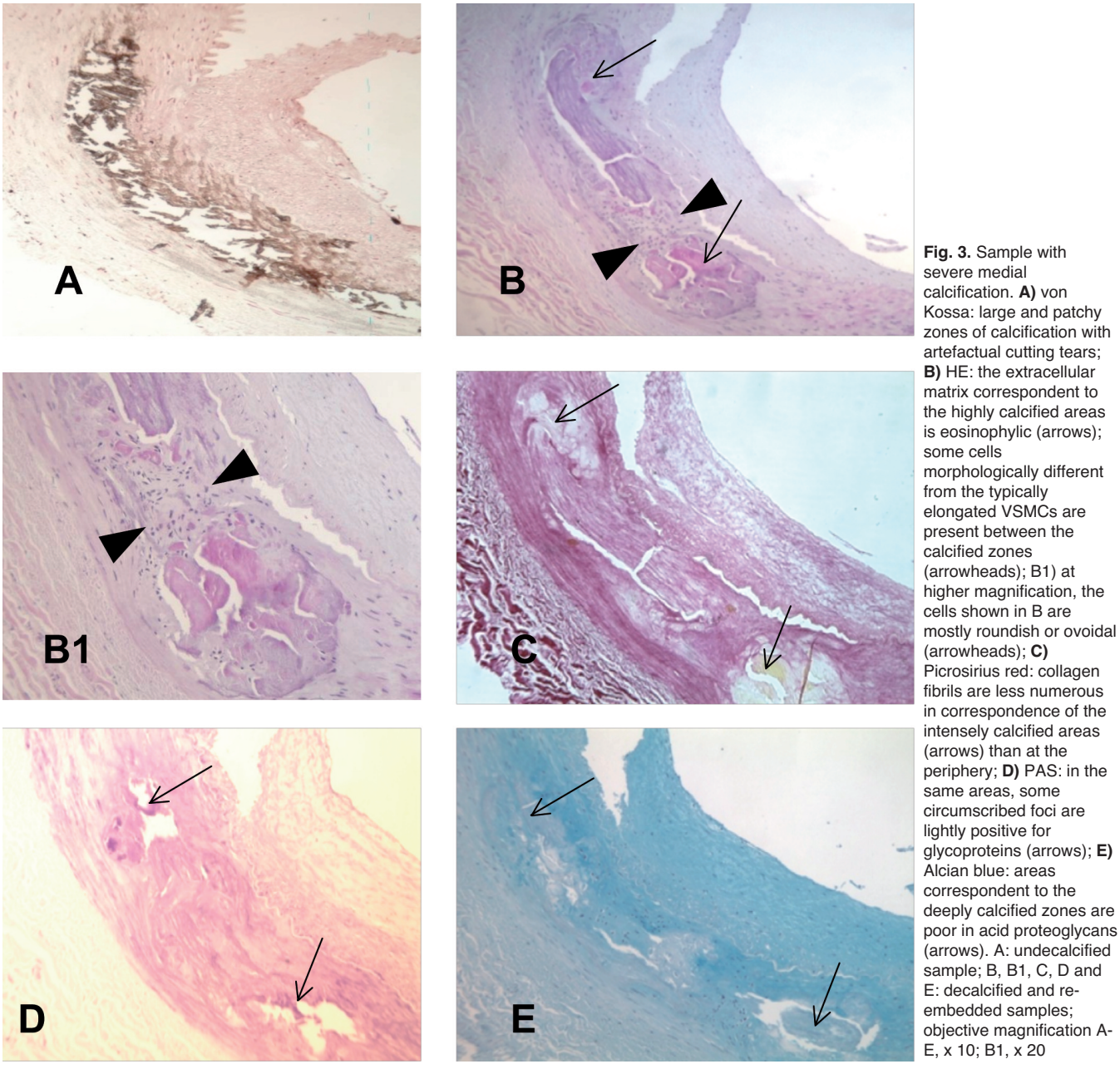

Fig. 3. Sample with severe medial calcification. **A)** von Kossa: large and patchy zones of calcification with artefactual cutting tears; **B)** HE: the extracellular matrix correspondent to the highly calcified areas is eosinophylic (arrows); some cells morphologically different from the typically elongated VSMCs are present between the calcified zones (arrowheads); B1) at higher magnification, the cells shown in B are mostly roundish or ovoidal (arrowheads); **C)** Picrosirius red: collagen fibrils are less numerous in correspondence of the intensely calcified areas (arrows) than at the periphery; **D)** PAS: in the same areas, some circumscribed foci are lightly positive for glycoproteins (arrows); **E)** Alcian blue: areas correspondent to the deeply calcified zones are poor in acid proteoglycans (arrows). A: undecalcified sample; B, B1, C, D and E: decalcified and re-embedded samples; objective magnification A-E, x 10; B1, x 20

therefore, had been de-paraffinized, decalcified and re-embedded, clearly showed that the previously calcified areas consisted of lightly eosinophylic amorphous material (Fig. 3B,B1), which was lightly stained by Picrosirius Red (Fig. 3C), PAS (Fig. 3D), and Alcian blue (Fig. 3E). A few roundish or ovoid cells, which could easily be distinguished with respect to the typically elongated VSMCs, were found in this material and between the calcified zones (Fig. 3B,B1).

A degenerated area near a highly calcified, ground substance-rich and cell-poor zone was selected for TEM investigation in the de-paraffinized samples with severe calcification that had been embedded in Epon after 'en bloc' staining with uranyl acetate (Fig. 4A). In such areas, several VSMCs were characterized by degenerative changes such as dispersed cytoplasmic vacuoles and decreased amount of myofilaments, this latter being roughly proportional to the increase in number and dimension of vacuoles (Fig. 4B-D). The finding of intact and uninjured mitochondria, which are very delicate organelles, in the proximity of the vacuoles in the cytoplasm and the dispersed chromatin in the nucleus, demonstrates that these degeneration elements are not artefactual (Fig. 4B,B1) but are part of degenerative changes (Fig. 4B-D). In contrast, distinctive ultrastructural apoptotic modifications, such as cell shrinkage, chromatin condensation along the nuclear periphery and margination of the nucleus, were not found. The media contained several extracellular, markedly electron-dense masses formed by bundles of filamentous substructures with a striated or banded pattern showing an axial periodicity of about 30 nm, demonstrating that they corresponded to fibrin. Fibrin bundles colocalized with calcific deposits (Fig 4 E,E1). Loosely arranged extracellular collagen fibrils, mixed with typical membrane-bound matrix vesicles, could also be found (Fig. 5A,A1). In the same context, characteristic structures consisting of electron-dense

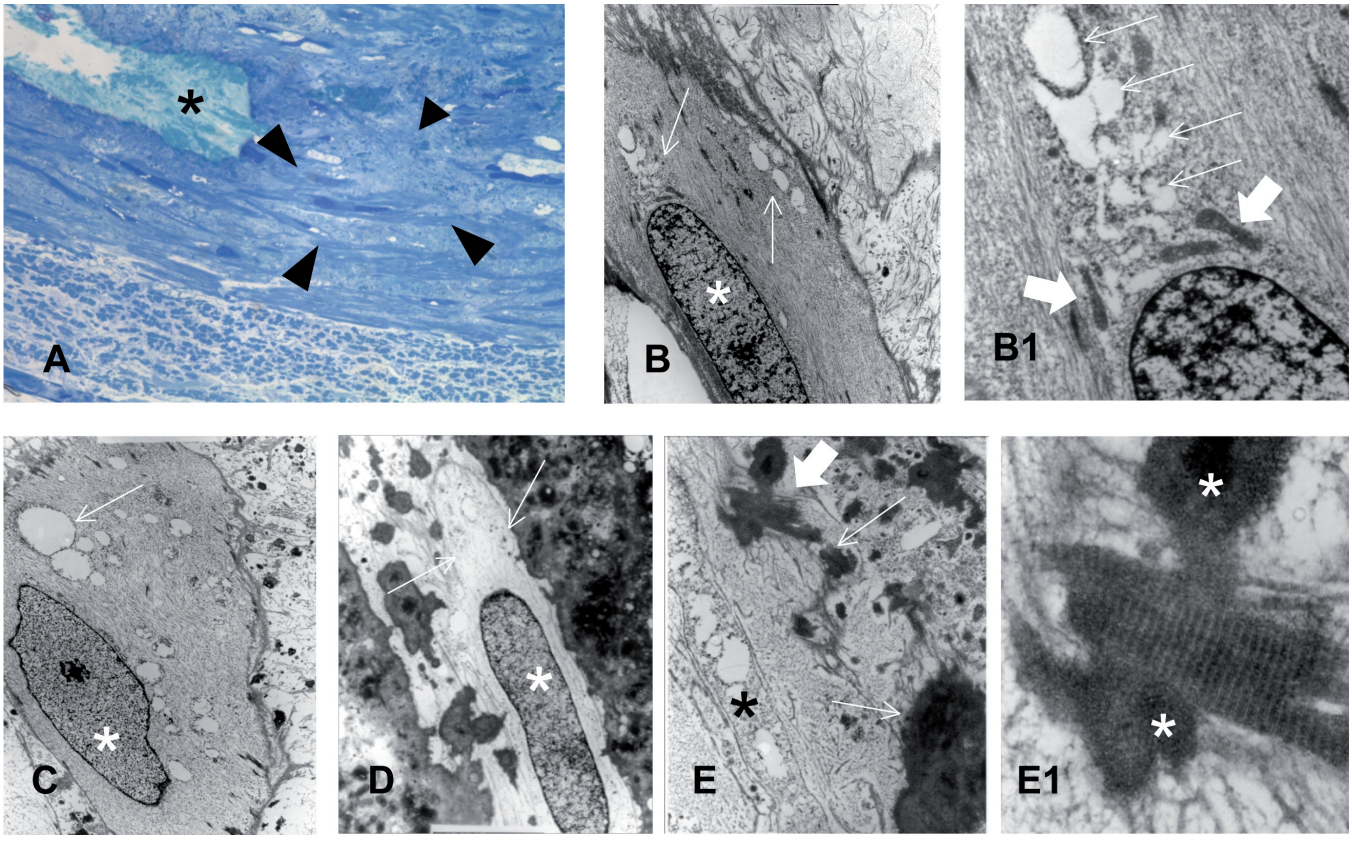

Fig. 4. Decalcified, Epon embedded samples with severe medial calcifications: **A)** the arrowheads point to the area selected for TEM investigation, near a zone with severe degree of medial degeneration (asterisk); semithin section stained with Azure II-Methylene Blue; objective magnification x 40. **B, C and D**) VSMCs containing cytoplasmic vacuoles (arrows): numbers and dimensions of vacuoles, lower in B and higher in D, are inversely proportional to the amount of myofilaments, higher in B and lower in D; nuclei show dispersed chromatin (asterisks); **B1)** detail of B: intact mitochondria (thick arrows) are recognizable in proximity of degenerated vacuoles (arrows); **E)** near a degenerated VSMC (asterisk), dense osmiophilic homogeneous masses, surrounded by (arrowhead) or embedded in (arrows) calcific deposits, are visible; **E1)** at higher magnification, the homogeneous material shown in E by the arrowhead is characterized by transverse striations of about 30 nm periodicity, which indicate that it corresponds to strands of fibrin; asterisks indicate calcific deposits. B, B1, C, D, E and E1: ultrathin sections stained with lead citrate; electron micrograph B, C, x 13,000; D, x 16,525; E, x 28,750; B1, x 30,000; E1, x 62,500.

concentric rings, corresponding to the so-called Liesegang rings, were recognizable (Fig. 5B,B1). These rings contained thin filamentous bands that were also found inside other roundish organelles, with variable areas of electron-density, and were recognizable as membranous structures (Fig 5C,C1). Roundish bodies, entirely consisting of membranous structures, very likely corresponding to degenerative and necrotic cell debris, could also be found (Fig. 5D,D1).

Discussion

The vascular samples were free from atherosclerotic lesions or intimal calcifications, so that pro-atherosclerotic risk factors could not be taken as responsible for the calcifications found in the media of our patients. This finding is in agreement with the fact that atherosclerotic plaques are more frequently found in large arteries, i.e. coronary artery, than in the peripheral ones (Moe et al., 2003; Gross et al., 2007). Patients with diabetes were deliberately excluded, since calcification of the media, especially in the lower extremities, and the consequent vasculopathy are commonly seen in these patients (Shanahan et al., 1999).

Within the media, calcifications displayed varying types of diffusion and degree of severity, ranging from a scattered and dot-like pattern limited to the media next to the adventitia in samples with light calcifications, to granular calcifications that formed approximately circumferential rings and gradually involved all the media in the moderate to advanced cases, and wide areas of consolidated calcium deposits in the most severe cases. In samples with moderate calcification, a gradient could be recognized within the tunica media, including an outer zone with granular extracellular calcifications, an intermediate zone with abundant extracellular matrix, and an inner zone with normally distributed VSMCs. In samples with severe calcification of the media, calcifications often aggregated and consolidated to form large, patchy calcium deposits. The calcification appears

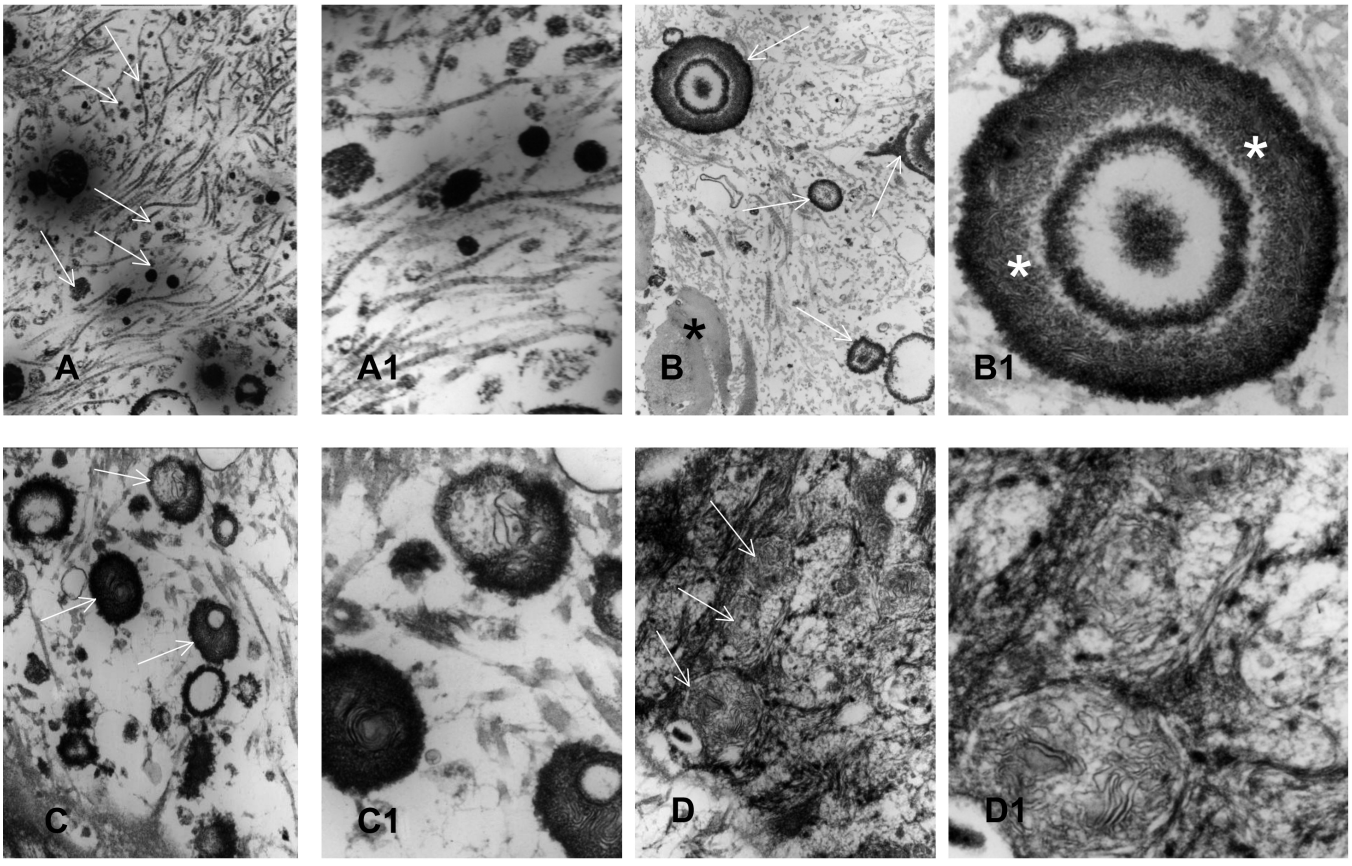

Fig. 5. A) Loosely distributed collagen fibrils mixed with matrix vesicles (arrows); **A1)** higher magnification of A showing matrix vesicles of various dimensions; **B)** electron-dense Liesegang rings (arrows); a fragmented elastic fiber is visible on lower-left (asterisk); **B1)** detail of B showing typical concentric Liesegang rings in which filamentous, membranous-like structures are recognizable (asterisks); **C)** several partially calcified, globular structures (arrows); **C1)** higher magnification of C, showing in detail the organic, membranous scaffold of the globular structures; **D)** nearly circular bodies consisting of almost concentric laminar threads (arrows), very likely corresponding to membranous fragments originating from cell necrosis; **D1)** detail of two globular bodies shown in **D**: the membranous nature of these bodies is clearly recognizable. Ultrathin sections stained with lead citrate; electron micrograph A, x 38,750; B, x 28,750; C, D, x 52,500; A1, x 62,500; B1, x 75,000; C1, D1, x 87,500

to progress from sites near the adventitia towards the intima, a finding already reported in rat arteries with experimental serum-induced calcification and considered to depend on the way of diffusion of the serum nucleating agent into the medial wall (Price et al., 2006). An approximately concentrical involvement of the tunica media was also found in diabetes-dependent calcification (Vattikuti and Towler, 2004) and was attributed to the supply of nutrients and mesenchymal progenitors the tunica adventitia provides to the tunica media through its microvascularization. Such functional spatial relationships would help to explain the circumferential pattern of medial artery calcification (Shao et al., 2005). These findings and considerations seem to be in contrast with the hypothesis of Jono et al. (2006) that in chronic kidney disease the factors that induce calcification originate in the blood lumen and diffuse to the media through the tunica intima.

In our samples, ossified areas were not found. However, besides zones of amorphous medial calcification, ossified areas were found in peripheral arteries of two of 21 diabetic patients who had undergone leg amputation (Shanahan et al., 1999). It is possible that, in the more severely damaged patients and in larger arteries, osteogenic proteins present in the extracellular matrix may induce bone formation through an osteoinductive process (Heliotis et al., 2009).

In samples with moderate calcifications, the VSMCs, especially those of the external and intermediate zones of the media, showed cytoplasmic vacuoles. Under the TEM, they were present in cells that, as normal, contained abundant myofilaments and dense bodies. Larger and more numerous vacuoles were found in the cytoplasm of severely degenerated cells, almost devoid of myofilaments. In cases with severe calcification, roundish or ovoid cells, morphologically different from the typically elongated VSMCs, were found in and between the intensely calcified zones. Under the TEM, these cells showed various degrees of degenerative changes, ranging from a few vacuoles in the cytoplasm to many large cytoplasmic vacuoles associated with lack of myofilaments. Several cells appeared to have undergone necrosis and disintegration. Preliminary immunohistochemical results have shown that, in patients with as well as without medial calcifications, VSMCs were generally positive to both α-SM-actin and the osteoblast differentiation factor RUNX2, as if they were transdifferentiating cells. However, the highly degenerated VSMCs found in patients with severe calcifications appeared to be dedifferentiated cells, being either lightly positive or negative to these proteins (Pisanò et al., 2009).

In samples with severe calcification, the necrotic cells were in contact with filamentous bundles, having a banded pattern and a periodicity well within the range of 19-35 nm, which is characteristic for fibrin and allows unequivocal identification of this substance (Ghadially, 1988). The occurrence in the wall of blood vessels of fibrin deposits, indicative of the so-called fibrinoid necrosis, was described in systemic malignant and pulmonary hypertension (Heath and Smith, 1978), and also in normotensive nephrectomized animals (Eto et al., 1978). Moreover, parallel bundles and threads of fibrin were localized in the intima, subintima and media of atherosclerotic vascular lesions (Bini et al., 1999). In our patients, the mechanism responsible for such a finding is not certain. Severe medial calcification may be responsible for increased vascular wall permeability and fibrin insudation into the media. Fibrin bundles colocalized with calcific deposits. It has been reported that, in valvular as well as vascular atherosclerotic calcification, fibrin appears to elicit both dystrophic and osteogenic responses (Bini et al., 1999; Rodriguez and Masters, 2009). Other *in vitro* studies have shown that fibrin has an inherent ability to promote valvular calcification (Benton et al., 2008) and that it is a useful scaffold base for modulating factors required for bone regeneration (Osathanon et al., 2009). By considering these reports, it cannot be excluded that fibrin might contribute to the calcification process, at least in those patients with severe medial calcification.

The more deeply calcified areas found in severely calcified samples had a low content of glycoproteins, proteoglycans and collagen fibrils. In general, independently of the severity of calcification, the extracellular calcified matrix revealed sparse collagen fibrils together with morphologically typical matrix vesicles (Bonucci, 2007). These vesicular structures may derive from cell death. VSMC necrosis can cause the release of membrane-bound fragments from the cytoplasm into the extracellular space. In the progression of the mineralization process, the appearance of calcified vesicles precedes the formation of substantial calcification of extracellular ground substance (Bonucci, 2007). Matrix vesicles mixed with collagen fibrils were already reported in a sample of inferior epigastric artery from a uremic patient (Moe et al., 2002), but neither the condition of the cells nor the type of cell death were described in this study. In another TEM study, samples of the inferior epigastric artery from a dialysis child with no evidence of calcification, as ascertained by von Kossa method, showed several VSMC damages, as increased electron density of nuclear heterochromatin, cell shrinkage, and/or vesicle release (Shroff et al., 2008), which were attributed to apoptosis and were considered an early event preceding overt calcification. However, since VSMCs with pycnotic nuclei did not have all the hallmarks of apoptosis or necrosis, the Authors suggested that apoptosis of contractile VSMCs might have unique features. In the same study, the extracellular vesicles of pediatric patients without calcification did not contain any evidence of hydroxyapatite nanocrystals, while vessels of other children with speckled calcification, as shown by von Kossa, had vesicles stained with high contrast, indicating the presence of mineral. The vesicles found in the extracellular matrix were consistent with their derivation from both apoptotic cells and budding from the plasma membrane.

Matrix vesicles have been shown to initiate calcification in cartilage, bone and other tissues. They originate from budding and pinching off from the plasma membranes of chondrocytes, osteoblasts, and odontoblasts. The regulation of matrix vesicles formation is not yet fully understood but it is thought that it can be associated with apoptotic cell death (Trion and van der Laarse, 2004). Apoptosis and apoptotic bodies have also been linked to vascular calcification in vitro. Human VSMCs spontaneously form multicellular nodules, where vesicle-like structures can be identified, and deposit calcium crystals in culture (Proudfoot et al., 1998). It has been shown that VSMC-derived apoptotic bodies are able to concentrate and crystallize calcium, suggesting that they might initiate vascular calcification in a fashion similar to matrix vesicles (Proudfoot et al., 2000).

It has been reported that, *in vivo*, TUNEL-positive VSMCs are more numerous in the tunica media of dialysis than of predialysis children (Shroff et al., 2008). They were seen in the von Kossa-positive regions (six out of 24 samples) and were attributed to apoptosis. Apoptosis, a physiological phenomenon counter-balancing cell proliferation, is characterized by cytoplasmic blebbing, chromatin condensation and DNA fragmentation. The TUNEL method, which recognizes DNA fragments, has been used extensively to detect apoptotic cells. However, damaged cells may undergo degeneration and necrosis, processes that are characterized by cell swelling and loss of the membrane integrity, followed by degradation of the DNA, which during the last stages of necrosis is digested into short, TUNEL-recognizable fragments (Bobryshev et al., 1997; Crisby et al., 1997; Yin et al., 2000). Studies carried out by light and TEM microscopy have shown that TUNEL-positive VSMCs had typical necrotic changes. In the intima of atherosclerotic lesions from carotid arteries Bobryshev et al. (1997) found a varying incidence of TUNEL-positive VSMCs, which, by TEM, appeared to die through necrosis rather than apoptosis. In carotid artery plaques, Crisby et al. (1997) have shown several TUNEL-positive cells, while TEM revealed that the majority of the VSMCs in the lesions were necrotic rather than apoptotic. Also TEM studies of *in vitro* cultures showed that necrotic cells were abundant within calcifying nodules, where necrotic cell debris and highly electron-dense rounded structures similar to matrix vesicles were also frequent (Proudfoot et al., 1998).

In our patients, VSMCs had the ultrastructural disintegration features typical of cells undergoing cell death by necrosis. In some circumstances, a light alteration, such as the increase of serum calcium and phosphorus frequently found in dialysis, may lead to apoptosis, but if the pathological stimulus becomes more severe and prolonged it can be high enough to cause necrosis (Hegyi et al., 1996). Thus, although cell death by apoptosis is considered a relatively frequent occurrence, most cells appear to die by necrosis. Debris of necrotic cells may originate matrix vesicles, or may also correspond to the filamentous-like, membranous structures, calcified or not, found in the extracellular spaces. Such structures may represent the organic frame that directs nucleation of calcium phosphate crystals. The formation of the typically circular, concentric inorganic deposits can be explained on the basis of a periodic precipitation of calcium phosphate on these organic structures, according to a mechanism similar to that which gives rise to the formation of the Liesegang rings in colloidal substances. In humans, analogous calcified bodies are usually formed in cysts, or in fibrotic, inflamed, or necrotic tissue of several organs (Onodera et al., 2009). To our knowledge no reports have documented the occurrence of Liesegang rings in media artery calcification.

In patients with chronic kidney disease an excessive amount of minerals in circulation is associated with increased risk of cardiovascular-related disease and mortality (Schoppet et al., 2008). Our results suggest that long-lasting accumulation of uremic toxic products causes a severe cellular insult that leads to VSMC death by necrosis and originates matrix vesicles and cell debris. The question whether arterial calcification is an active biological process or a merely passive precipitation of inorganic ions is still under debate (Duer et al., 2008). The active hypothesis is supported by the transformation of VSMCs into osteoblast-like cells, which express various bone-related proteins, and lay down a bone matrix that eventually calcifies (Moe and Chen, 2004). The passive hypothesis is based on the concept that, in an appropriate microenvironment, calcium and phosphate physicochemically precipitate in areas of advanced tissue degeneration or necrosis within the vascular wall when the physiological calcium phosphate solubility threshold is exceeded (Hofbauer et al., 2007). This largely extracellular process occurs in association with membrane debris. Our results, not necessarily in contrast with those supporting the phenotypic transformation of VSMCs into osteoblast-like cells, are in agreement with a unifying hypothesis of vascular calcification, that combines both active and passive mechanisms (Hofbauer et al., 2007). Under disturbances in mineral metabolism, VSMCs can acquire an osteoblastic phenotype and osteogenic properties; matrix vesicles and apoptotic bodies from these cells form the nidus for calcification. When VSMCs are severely damaged, they undergo necrosis with liberation of cell debris and matrix vesicles. In both cases, these last, and the remains of the necrotic tissue, may represent the scaffold permitting the precipitation of the mineral substance.

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