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MOLECULAR AND CELLULAR MECHANISMS OF VASCULAR CALCIFICATION: PATHOGENESIS AND TREATMENT

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1. ABSTRACT

Vascular calcification is a significant contributor to cardiovascular risk in chronic kidney disease (CKD) patients, and its extent and severity has been correlated with mortality in several studies.

Hyperphosphatemia predisposes these patients to early and progressive vascular calcification: it appears to be involved in a number of mechanisms that trigger and promote the progression of this active and cell-mediated pathological process, in which vascular smooth muscle cells (VSMCs) residing in the tunica media of blood vessels are the main cell type actively involved.

We developed an *in vitro* model to elucidate the molecular and cellular mechanisms involved in the pathogenesis of vascular calcification: in particular, we challenged rat VSMCs for 7-15 days with high Pi (inorganic phosphorous) with the purpose to reproduce *in vitro* the same pathological process that occurs in CKD patients *in vivo*. We investigated the high Pi-induced calcium deposition and the modulation of different cellular biological processes (apoptosis, autophagy and VSMC osteoblastic differentiation) through molecular biology, proteomic and immunohistochemistry analysis.

First of all, we studied the modulation of osteonectin (SPARC), a major non collagenous protein of bone matrix that is associated, generally, with remodeling of tissues, mineralization and pathological responses to injury. Since there are controversial results regarding its role during the process of vascular calcification, we investigated osteonectin expression both *in vitro* and *ex-vivo*, and the results suggest a pro-calcifying role of this protein in the process of vascular calcification.

Then, we developed an experimental strategy to delay the progression of calcium deposition in our *in vitro* model. We studied the potential effect of repeated and short time suspension of high Pi treatment (process that we called “Wash Out”) on the progression of calcium deposition, trying to reproduce the same temporal decrease of Pi levels that occurs in CKD patients treated with haemodialysis. Surprisingly, we discovered that it is sufficient a temporary total absence or partial decrease in Pi concentration under a so called “trigger threshold” during the process of calcification to obtain a substantial inhibition of calcium deposition. The molecular and cellular pathways involved in this protective action are

apoptosis, VSMC osteoblastic differentiation and autophagy: the formers are partially inhibited, while the latter is incremented after the “Wash Out” treatment.

Finally, we investigated the mechanism of action of two drugs CKD patients are treated with in the attempt to reduce hyperphosphatemia and to contrast secondary hyperparathyroidism, respectively: Lanthanum Chloride ($LaCl_3$) and Calindol. We demonstrated that these compounds significantly delay the progression of high Pi-induced VSMC calcium deposition, but with different mechanisms of action: both of them delay VSMC osteoblastic transformation, and, in particular, lanthanum chloride preserves VSMC lineage markers expression and partially prevents VSMC apoptosis, whereas calindol increases the expression of an anti-calcifying protein.

These *in vitro* discoveries can suggest that vascular calcification is a multifactorial process that involves different cellular and molecular pathways, and that it is of relevant importance to control as more as possible phosphate levels in CKD patients, even with diet, because high-Pi is the most dangerous key regulator of vascular calcification in end stage renal disease pathology.

2. INTRODUCTION

2.1 Definition and clinical significance of vascular calcification

In the past decade, the prevalence, significance and regulatory mechanisms of vascular calcification have gained increasing recognition. Over a century ago, pathologists recognized atherosclerotic calcification as a form of extraskeletal “ossification”. One major mechanism in the development of vascular calcification is similar to that of bone formation: in particular, vascular smooth muscle cells (VSMCs) residing in the tunica media of blood vessels undergo osteogenic differentiation into phenotypically distinct osteoblast-like cells. This phenotypic transformation involves bone morphogenetic proteins and potent osteochondrogenic transcription factors, and it is triggered and modulated by a variety of inflammatory, metabolic and genetic disorders, particularly dyslipidemia, chronic kidney disease (CKD), diabetes, hyperparathyroidism and osteoporosis (1).

Calcium phosphate deposition, mostly in the form of apatite, is the hallmark of vascular calcification and can occur in the blood vessels, myocardium and cardiac valves. Calcium phosphate deposits are found in distinct layers of the blood vessels and are associated with specific pathologies. Intimal calcification is observed in atherosclerotic lesions (2), whereas medial calcification is common to the arteriosclerosis observed with age and diabetes, and is the major form observed in end-stage renal disease (ESRD) (3). In ESRD patients, both intimal and medial calcification occurs, but arterial medial calcification is by far the most prevalent. Thus, both intimal and medial calcifications may contribute to the morbidity and mortality associated with cardiovascular disease, and are likely to be major contributors to the 10–100 fold increase in cardiovascular mortality risk observed in ESRD patients (4).

The exact mechanism and process of calcification within the arterial wall in ESRD is not yet completely understood. There is strong evidence that vascular calcification is closely associated with serum levels of calcium, phosphate and calcium x phosphate product (5, 6).

Two different mechanisms are proposed to explain the relationship between calcium and phosphate disorders and vascular calcification: a passive one, the direct calcium-phosphate precipitation in the vasculature, and an active one, that induces the expression of bone-associated genes in VSMCs, that acquire the phenotype of bone-forming (osteoblast-like) cells (5, 7).

2.2 Types of vascular calcification process

In general, vascular calcification can be categorized into four different types: intimal calcification (atherosclerotic), medial calcification (calcification in tunica media), valvular calcification and vascular calciphylaxis (8, 9).

Atherosclerotic calcification is a type of dystrophic calcification which can be characterized by cellular necrosis, inflammation, and lipoprotein/phospholipid complexes. In association with atherosclerotic plaques and old myocardial infarcts, lipid complexes, which are derived from cellular membranes, and serum lipoproteins nucleate calcium deposition. Endothelial cell dysfunction drives additional lipid deposition through providing a thrombogenic surface which, in turn, provides fibrin and platelet derived phospholipids. Following the degenerative tissue calcification, calcified cartilage forms via a vascular remodeling process through endochondral ossification process. It is a patchy and discontinuous process that involves macrophages and VSMCs in lipid-rich regions (8, 10).

Medial calcification usually occurs in the absence of lipid and is associated with α-smooth muscle actin-positive VSMCs (9). It is an intramembranous ossification process of the arterial tunica media, which resembles bone formation and odontogenesis (8). It does not require any cartilaginous precursor, and BMP2/Msx2 dependent signaling is a central feature of the mineral formation (8). It is focal in distribution, organized along the elastic lamellae, and is almost exclusively associated with VSMCs. Moreover, it has been reported by multiple researchers that vascular calcification is initially triggered by matrix vesicles in association with fibrillar collagen extracellular matrix (8, 11).

The differences between intimal and medial calcification imply different etiologies; however, a common feature of both forms of calcification is the presence of VSMCs (12).

Cardiac valve calcification is known to be induced by mechanical stress and inflammation, which is followed by dystrophic mineralization and intramembranous ossification in association with endochondral ossification (although to a lesser extent compared to that of the atherosclerotic calcification) (8, 13). It is believed that aortic valve calcification is strongly related to aging in many cases where, degenerative calcification, which is the most common cause of aortic stenosis in patients older than 70 years, is observed (13).

Vascular calciphylaxis, also known as calcific uremic arteriolopathy, usually occurs in patients with secondary hyperparathyroidism and renal insufficiency, especially those

withend-stage renal hemodialysis (14, 15). From a biochemical point of view, it occurs when the physiological calcium-phosphate solubility (60 mg^2/dl^2) level is exceeded, exhibiting widespread deposition of amorphous calcium phosphate (10). However, certain case reports describe the occurrence of the syndrome in patients with normal renal and parathyroid function, and no abnormalities in calcium and phosphorous level (14).

2.3 Vascular calcification is an actively regulated process

For many years, vascular and soft-tissue calcification were thought to occur predominantly by a passive, unregulated physicochemical mechanism and to represent a degenerative, irreversible process of aging (16). However, as early as the 19th century, Virchow described bone-like structures in the vasculature, which provided the first clue that vascular calcification may be a regulated process. Converging evidence from *in vivo* analyses of histopathology and gene expression in human vessels, as well as animal knockout models, and analysis of human genetic defects all support the hypothesis that vascular calcification is an active, cell-mediated process (17). Over the past ten years, our understanding of molecules and processes that regulate ectopic calcification has grown exponentially. Much of our understanding comes from identification of genes through linkage or targeted deletion studies that cause human and/or mouse ectopic calcification disorders, respectively (18).

Vascular calcification, like bone formation, is a highly regulated process, involving both inductive and inhibitory processes (19). Cells derived from the tunica media, including VSMCs, adventitial fibroblasts, and pericytes, undergo osteochondrogenic differentiation and matrix mineralization under the appropriate conditions *in vitro* (20-24). These studies suggest that cell-mediated processes tightly control procalcific and anticalcific mediators in the artery, so that ectopic calcification is normally avoided. Under pathological conditions, this balance is upset and leads to ectopic mineralization.

Many studies have shown that the mineral deposited in the vessel wall is basic calcium phosphate in apatitic form, some of which is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], the same crystal found in bone. Moreover, VSMCs *in vivo* have been observed to bud matrix vesicles from their plasma membrane, first described in chondrocytes and osteoblasts during developmental osteogenesis, that form a microenvironment capable of concentrating

calcium and phosphate, thus allowing crystal nucleation to occur (12). However, while skeletal mineralization is a regulated process induced by complex, well-timed developmental cues, vascular calcification is a pathological process, occurring in response to dysregulated environmental cues.

Under normal conditions, inhibitors of soft-tissue mineralization, such as matrix γ-carboxyglutamic acid protein (MGP), are expressed locally within the vessel wall while others, such as fetuin-A, are present in the circulation. Down-regulation or perturbation of these proteins leads to a phenotypic transformation of VSMCs into osteo⁄chondrocytic-like cells that have the capacity to modulate the mineralization process (1).

Local VSMCs, as well as circulating mesenchymal stem cells, local pericytes and fibroblasts that exhibit multilineage potential, may transdifferentiate into osteo/chondrocytic cells in the arterial wall and orchestrate bone formation and calcification. The osteo/chondrocytic conversion of VSMCs and stem cells both *in vitro* and *in vivo* is accompanied by up-regulation of Cbfa1/Runx2, osterix, Msx2 and Sox9, transcription factors that are centrally involved in chondrocyte maturation and osteoblastic differentiation (1).

Accumulating evidences suggest that multiple factors, such as hypertension, reactive oxygen species, advanced glycation end products, lipids, inflammatory proteins, such as tumor necrosis factor-α, and potentially others, like high phosphorous (Pi) levels and yet unidentified damage-inducing agents, initiate osteo/chondrocytic conversion and matrix vesicles release in VSMCs (25).

The current major theories and regulators of vascular calcification include 1) loss of inhibition, 2) induction of osteochondrogenesis, 3) apoptosis, 4) abnormal calcium and phosphate homeostasis, 5) circulating nucleation complexes/paracrine factors derived from bone and 6) matrix degradation (18) (Fig. A).

Studies in animal models have provided important insights into the pathogenesis of vascular calcification, but approaches to stop progression or even reverse the pathology are in their infancy. One key area for further investigation is the role of circulating proteins in regulating calcification, either by effects on maintenance of calcium phosphate homeostasis in solution or by direct effects on vascular cell function.

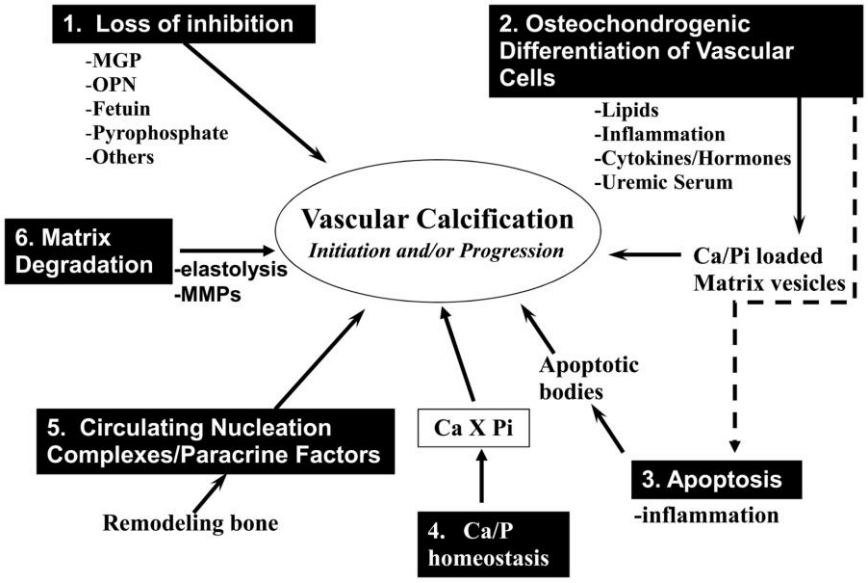

Figure A: Cellular and Molecular Mechanisms of Vascular Calcification
Six different mechanisms that have been proposed to regulate the initiation or progression of vascular calcification (18).

2.3.1 Inducers of vascular calcification

Core Binding Factor α-1 (CBFA-1)

Cbfα-1 is a pivotal transcriptional regulator of osteogenesis and plays a crucial role in the lineage determination of connective tissue progenitor cells. VSMCs and osteoblasts are derived from mesenchymal precursor cells, and Cbfα-1 can induce osteoblastic differentiation in both cell types. Cbfα-1 is up-regulated in calcified human atherosclerotic plaques and human VSMCs *in vitro* along with other osteoblastic and chondrocytic markers (e.g. osteocalcin, alkaline phosphatase and bone sialoprotein) that are under its regulation (26). Expression of this plethora of mineralization-regulating proteins by VSMCs may represent an attempt by the cells to further regulate the calcification process; however, the exact role of these multifunctional proteins in the vasculature remains unknown (12).

Bone Morphogenetic Protein (BMP)-2 and -4

These proteins are inflammatory mediators in vascular endothelium responsive to disturbed flow, increased oxidative stress and inflammation. Increased BMP activity enhances atherogenesis and vascular calcification (27, 28).

The BMPs belong to the transforming growth factor (TGF)-β superfamily and elicit their response via the so-called type I and II receptors. BMP-2 and -4 interact with the activin-like kinase receptor ALK2, ALK3 and ALK6, which are type I receptors that form complexes with the BMP type II receptor (BMPRII). In canonical BMP signaling, the receptors phosphorylate specific regulated (R)-SMAD proteins, which translocate into the nucleus and regulate gene transcription. SMAD1/5/8 mediate BMP-signaling, whereas SMAD2/3 mediate TGF-β signaling (29).

The high BMP activity was associated with a remarkable increase in aortic expression of osteogenic markers and calcification. Increased BMP inhibition, as mediated by a MGP transgene, limited these changes (29).

Other pro-calcific proteins

Muscle Segment Homeobox-2 (MSX2)

It appears to be a critical gene in vascular calcification up-regulated by the action of BMP-2. MSX2 is a member of the homeobox gene family and plays an important role in bone formation and temporal spatial timing of osteoblast differentiation. The mineralization process of tunica media calcification is akin to intramembranous bone formation, and studies demonstrate MSX2 expression and function in vascular media calcification. The effect of MSX2 is through up-regulation of osterix (Osx), a global transcriptional regulator of mineralization and osteoblast differentiation (30).

Osterix (Osx)/Sp7

It is a member of the Sp1 transcription factor family, plays an essential role in bone formation and osteoblastogenesis. Although Osterix has been shown to be induced by BMP2 in a mesenchymal cell line, the molecular basis of the regulation, expression and function of this protein during osteoblast differentiation is not fully understood. To date, the target genes of osterix have not been identified. Interestingly, it promotes osteoblast differentiation of Runx2-deficient mesenchymal cells in association with up-regulation of several genes, which are not up-regulated by Runx2. Therefore, identification of the genes that are regulated by Osterix and that play critical roles in osteoblastogenesis may advance the understanding of the molecular mechanism by which BMP2 regulates bone formation

and osteoblastogenesis and may contribute to developing therapeutic agents for bone diseases (25).

2.3.2 Inhibitors of vascular calcification

Matrix Gla Protein (MGP) and Growth Arrest Specific Gene 6 (Gas-6)

They are two particularly important Vitamin-K Dependent Proteins (VKDPs), and their roles in vascular biology are just beginning to be understood. Both are produced by VSMCs and function to protect the vasculature: MGP prevents vascular calcification and Gas-6 affects VSMCs apoptosis (31).

Together, these proteins constitute a new mechanism of local vascular regulation, where the blood vessel defends itself against injury and participates in self-repair. A failure of these local mechanisms might be an important first step in a cascade of events culminating in vascular calcification, and supports the notion that vascular calcification is an active, regulated process.

To become biologically active, both MGP and Gas-6 undergo carboxylation, a process that occurs at the blood vessel level (31). Like hepatic carboxylation, this peripheral carboxylation is inhibited by the administration of warfarin, yet whereas warfarin's anticoagulant effect is well known, its effect on the vasculature is less certain.

Produced by many cells types and binding to one of three receptors (Tyro3, Axl, or Mertk), **Gas-6** has widespread physiologic roles, important in inflammation, renal disease, sepsis, and neoplasia (32). Gas-6 produced by VSMCs seems to have a pro-survival effect: by binding to its receptor **Axl**, it stimulates the anti-apoptotic protein Bcl-2 and inhibits the proapoptotic protein caspase 3, ultimately conferring a state of longevity to the cell (33). Indeed, recent *in vitro* studies have shown Gas-6 prevents vascular calcification. In a model of phosphate-induced vascular calcification, Son et al. have shown that Gas-6 prevents vascular smooth muscle cell apoptosis and calcification in a dose-dependent manner (34). Elevated phosphate levels directly suppressed Gas-6 activity (35). These findings raise fascinating questions about the mechanisms of vascular calcification. As originally suggested by Proudfoot et al., whose important work showed that vascular smooth muscle cells develop apoptotic bodies before calcium crystals, apoptosis may be the critical first step, with calcification as a secondary phenomenon of dying cells (36).

MGP also has important functions in vascular biology. It is produced in bone and VSMCs, and prevents vascular calcification. Its clinical importance is evidenced by the development of widespread and extensive vascular calcification in MGP knockout mice, which is prevented if arterial MGP expression is genetically restored (37). In humans, a rare genetic disorder resulting from mutations in the MGP gene, Keutel syndrome, is associated with extensive soft tissue calcification and vascular calcification (38). In postmortem evaluation, calcium deposition is primarily located within the media of the vessel wall, specifically the elastic interna, with little intimal involvement. The potential role of MGP polymorphisms in vascular calcification is not fully known yet raises interesting questions about genetic susceptibility to vascular calcification (39). MGP plays a crucial role in preventing the calcification of arteries: paradoxically, MGP expression is up-regulated in calcified human plaque, probably through a negative feedback mechanism in response to calcification (12).

Despite the clinical importance of MGP in preventing vascular calcification, its exact mechanism of action remains unknown. MGP complexes with ambient calcium, preventing calcium supersaturation and crystallization within vessel walls and, by binding to hydroxyapatite crystals, inhibits their crystalline growth. MGP also inhibits VSMCs from dedifferentiating into osteoblast-like cells, a well-established mechanism for vascular calcification. Recent studies suggest that the activity of MGP is affected by BMP-2: higher levels of MGP will inhibit BMP-2 activity (40, 41).

Namely, as part of the VKDP family, both Gas-6 and MGP must be converted to their functional forms by undergoing carboxylation. Thus, in addition to the expression of these proteins, the structural form determines functional activity.

The process of converting VKDPs to their biologically active forms requires the carboxylation of glutamic acid residues, a complex and incompletely understood process involving multiple enzymes (31). As a final step, γ -glutamyl carboxylase (GGC) facilitates the addition of a CO_2 molecule to the γ-carbon of glutamic acid, forming γ-carboxyglutamic acid. This requires the presence of the reduced form of vitamin K. Because vitamin K naturally occurs in the oxidized form, it must be converted into a reduced form, a reaction catalyzed by vitamin K epoxide reductase (VKOR). In this process of carboxylation, vitamin K is returned to its oxidized state, and a cycle of vitamin K reuse ensues. Warfarin, which shares a common ring structure with vitamin K, interferes with VKOR, interrupts vitamin K recycling, prevents

further γ-carboxylation, and ultimately leads to a deficiency of active VKDPs. Ultimately, the complexity of the carboxylation process, with multiple enzymatic reactions and many polymorphisms of participating gene products, leads to a wide range of VKDP phenotypes, and, partly, explains the variation in individual susceptibility to warfarin.

Important studies by Murshed et al. have shown that, although the liver makes MGP, such hepatic production does not protect against vascular calcification; rather, the protein must be locally produced within the vasculature (37). This tissue localization might explain why serum levels of MGP do not correlate with its biologic activity. In addition, the process of carboxylation differs between the liver and the periphery. The activity of VKOR, the enzyme responsible for vitamin K recycling, is three times higher in vascular smooth muscle cells than in the liver, suggesting that peripheral carboxylation may be more dependent on adequate stores of vitamin K (31).

Schurgers et al. have illuminated the critical importance of post-translational carboxylation of peripheral VKDPs (42). Although both normal and calcified vessels express MGP, normal vessels have a predominance of carboxylated MGP, whereas calcified vessels have a predominance of the uncarboxylated version (42). It seems that the ratio of carboxylated to uncarboxylated protein ultimately determines biologic activity of both MGP and Gas-6 (43).

These findings suggest a potential mechanism: to help protect blood vessels from injury, vascular smooth muscle cells continuously produce MGP and Gas-6, which then undergo carboxylation. The carboyxlated proteins form a defense against injury: MGP preventing crystalline supersaturation and Gas-6 regulating both apoptosis and migration of smooth muscle cells into areas of injury. Failure to activate these proteins leads to vascular injury.

In summary, Gas-6 and MGP provide local protective mechanisms, influencing cell death and calcification, and support the notion that the blood vessels themselves actively participate in their own defense (31).

Osteonectin (ON)

Osteonectin, also named SPARC (secreted protein, acidic and rich in cysteine), is a widely expressed profibrotic protein with pleiotropic roles. It is a calcium-binding matricellular protein present in skeletal tissue, where, rather than playing structural roles, primarly serves as biological modulator (44). The function of osteonectin in the skeleton relates to bone cell differentiation, control of remodelling and maintenance of bone mass (45). SPARC-deficient

mice have decreased bone formation and decreased osteoblast and osteoclast surface and number, which leads to a decrease in bone formation and remodeling, with a negative bone balance that causes profound osteopenia and severe cataract formation (46).

Beyond bone, osteonectin has been found in many tissue undergoing morphogenesis or remodelling, where it is expressed and secreted by different cellular types such as fibroblasts, endothelial cells, VSMCs and tumour cells.

In different tissues, osteonectin is able to regulate cell migration, cell proliferation, tissue morphogenesis and tissue repair even if its effects are cell type-dependent (44, 47). For example, the regulatory role of osteonectin in angiogenesis is supported by the increase of its expression in the vasculature during physiological and pathological angiogenesis and by the modulatory activity that this protein exerts on growth factors involved in the angiogenesis process such as VEGF, FGF2 and TGF-β (48). Besides angiogenesis, there are evidences that link osteonectin to obesity and diabetes mellitus. In fact, osteonectin is associated with diabetes complications such as diabetic retinopathy and nephropathy, conditions that are ameliorated in the SPARC-knockout mouse model and, as a regulator of the extracellular matrix, osteonectin also contributes to adipose-tissue fibrosis. (49). Regarding tumours, osteonectin modulate the interaction between tumour cells and the normal surrounding cells, affecting proliferation, survival and migration of cancer cells, tumour invasion and metastasis (50).

Regarding vascular calcification, osteonectin is present in VSMCs (51) and its expression has been found to be modulated in calcified arteries (52, 46, 53, 54). Moreover, osteonectin is also one of the most potent inhibitors of hydroxyapatite crystal formation *in vitro* (55, 56), but, despite this action, osteonectin deficient mice do not exhibit arterial calcification (57). Thus, osteonectin is expressed in VSMCs, but its role in vascular calcification is not well elucidated.

Osteonectin expression has been investigated predominantly in *ex vivo* studies on human arteries. Shanahan et al demonstrated that VSMCs in normal arteries express SPARC at high levels, however, in Mönckeberg's Sclerosis (MS) arteries (tunica media calcification), there was a significant reduction in the level of this protein expression. The high expression of SPARC in the normal vasculature and its absence in MS lesions suggest that its loss may

promote mineralization (52). However, Bini et al demonstrated the presence of osteonectin in association with large calcifications in atherosclerotic plaques (58).

The lack of knowledge on osteonectin does not allow to define its specific role. Further investigations are needed to precise its utility in calcification assessment.

Other anti-calcific proteins

Bone Morphogenetic Protein -7 (BMP-7)

It is an important regulator of skeletal modeling, restores skeletal anabolic balance, reduces serum phosphate levels, and, thereby, also reduces vascular calcification in experimental animal models of CKD, highlighting the importance of maintaining a balance in signaling in the vasculature during development and in disease (12).

Fetuin-A

All extracellular fluids, even under normal circumstances, are saturated with respect to calcium and phosphate, suggesting that potent inhibitors of vascular calcification are normally circulating to prevent ectopic soft-tissue calcification. The serum protein fetuin-A (a-2-Heremans–Schmid glycoprotein) is a key component of VSMCs-derived vesicles. It is a very potent inhibitor of apatite crystal formation in solutions containing calcium and phosphate and thus serves to maintain the solubility of calcium in plasma (12). Fetuin-A can inhibit apoptosis and enhance phagocytosis, and its incorporation into vesicles completely abrogates their ability to calcify.

Inflammation reduces fetuin-A synthesis, and it has been shown that, in hemodialysis patients, the fetuin-A levels are significantly lower than in healthy controls and are inversely related to C-reactive protein (59, 60). At sites of vascular damage, it is taken up by VSMCs, incorporated into intracellular vesicles and then released within matrix vesicles, where it potently inhibits mineral nucleation.

Hence, fetuin-A may inhibit the pro-calcific effects of mineral imbalance in normal arteries, and reduced levels in CKD contribute to induction of calcification (12).

Osteopontin (OPN)

OPN may affect calcification by stimulating resorption: by binding to a_vb_3 integrin on osteoclasts, it leads to a decrease in cytosolic calcium, resulting in osteoclast activation to a

resorptive phenotype (61). This binding may also promote resorption of ectopic calcification by inducing expression of carbonic anhydrase isoenzyme II (CA II) in macrophages, which is key in creating the acidic environment required for resorption (12).

Although OPN is not present in most normal soft tissues, it is abundant at sites of ectopic calcification in human atherosclerotic lesions, diabetic arteries, uremic arteriolopathy and valves. OPN has a high content of aspartic acid residues and is highly phosphorylated on serine and threonine residues: this structure enables OPN to bind to hydroxyapatite and calcium ions, and, thus, to physically inhibit crystal formation and growth *in vitro* (62).

2.3.3 Other regulators

The role of pH in promoting or inhibiting calcification is also important; crystal growth is favored in an alkaline medium. The **carbonic anhydrase isoenzyme II** (CA II) knockout mouse develops an age-dependent medial calcification of small arteries in a number of organs, with the male genital tract developing the most extensive arterial calcinosis (63). CA II deficiency in humans causes a rare autosomal recessive disorder characterized by osteopetrosis, renal tubular acidosis and cerebral calcification (64).

Perturbation of the pathway involved in the generation of pyrophosphate by inactivation of the enzyme **ecto-nucleotidepyrophosphatase/phosphodiesterase 1** (ENPP1) leads to ossification of the aorta. The mouse knockout for ENPP1 develops up-regulated alkaline phosphatase, decreased expression of OPN, increased calcification of aortic smooth muscle cells and chondrogenesis in mesenchymal precursors. In humans, mutations in ENPP1 cause infantile idiopathic arterial calcification, a condition in which the internal elastic lamina of muscular arteries calcifies resulting in death usually within the first year of life (65, 66).

Finally, there are a number of other factors that may specifically contribute to the procalcific environment observed in CKD patients. Parathyroid hormone can cause hypercalcemia, and active vitamin D3 (1,25-di-hydroxy vitamin D3), which is routinely used in the management of secondary hyperparathyroidism, itself contributes to ectopic calcification in both animal models and *in vitro*. However, the role these factors play in calcification is likely to be complex as they may have both concentration and temporal effects on both bone and vascular function with both too much, or too little, being harmful (12).

2.4 Vascular calcification and chronic kidney disease (CKD)

The clinical condition associated with major severe vascular calcification is end-stage renal disease, in which evidence points to inorganic phosphate (Pi) as central regulator. ESRD patients are often hyperphosphatemic, defined as a phosphate concentration higher than 2 mM, compared with 1–1.5 mM in healthy patients (25).

Uremic toxins present in the uremic milieu, such as a mineral imbalance, and the disruption of circulating and cellular inhibitors of calcium and phosphate precipitation induce VSMCs apoptosis, and vesicle release resulting in mineral nucleation and deposition of hydroxyapatite (1).

Among the 25 million American patients with stage 2–5 chronic kidney disease (CKD), cardiovascular disease causes a disproportionately high mortality risk. Patients with CKD are more likely to die (often of cardiovascular causes) than to progress to dialysis (67). The risk of death is especially high in late-stage kidney disease; a 30-year-old patient with end-stage renal disease faces an equivalent risk of death to a 90-year-old without CKD (18). CKD patients have a 30-fold higher mortality than the general population despite adjustment for traditional cardiovascular risk factors, such as diabetes mellitus and hypertension (68).

Recent epidemiological studies have shown that even minimal alterations in renal function (as evidenced by a reduced glomerular filtration rate, presence of microalbuminuria and elevated serum phosphate levels in the general population) are associated with a high cardiovascular risks (69).

Vascular calcification is a significant contributor to cardiovascular risk in CKD patients, and its extent and severity has been correlated with mortality in several studies (12).

The precise pathophysiology of vascular calcification in end-stage renal disease is unknown, but risk factors include age, hypertension, time on dialysis, and, most significant, abnormalities in calcium and phosphate metabolism (70-72).

Calcification of arteries occurs in the intima in association with atherosclerosis, where it may contribute to plaque formation and rupture, and in the media, where it causes vascular stiffening.

Although a combination of intimal and medial calcification may occur in patients with CKD, either process may occur independently of the other, and, at least in adolescents and young adults with CKD, the vascular ‘calcium load’ is almost exclusively medial. Increased arterial

stiffness is mechanistically linked with systolic hypertension, left ventricular hypertrophy, and reduced coronary perfusion, and is a significant independent predictor of mortality (53). CKD is associated with fatal cardiovascular consequences in part due to ectopic calcification of soft tissues particularly arteries, capillaries and cardiac valves.

As above mentioned, an increasing body of evidence from experimental studies and *in vivo* data suggest that (I) a mineral imbalance with hyperphosphatemia and high-circulating calcium x phosphate product, (II) a deficiency of systemic or local calcification inhibitors, (III) death or damage of vascular smooth muscle cells, and/or (IV) phenotypic transformation of VSMCs to osteo/chondrocytic cells may all act in concert to initiate and sustain vascular calcification (12).

However, in CKD a mineral imbalance is central to this damage with elevated phosphate inducing expression of Runx2 and osterix in VSMCs. More significantly, apoptotic bodies and matrix vesicles similar to those that nucleate mineral in bone are released from dying and damaged VSMCs and nucleate mineral in vascular tissues (36).

The devastating effects of CKD on the vasculature are the net result of multiple pathogenic mechanisms that overwhelm natural defenses against calcification. Circulating toxic elements that cause VSMC damage and death accumulate, while calcification inhibitors are decreased both locally and systemically (12).

Studies with genetically altered mice have identified both local and systemic calcification inhibitors that act to maintain VSMC differentiation or regulate vesicle properties. However, for many of these proteins, the mechanisms and sites of action are still under investigation. In particular, it is unclear whether factors present in the circulation have an inhibitory role there and whether circulating levels of these proteins influence or are indicative of underlying disease processes in individual patients. A greater understanding of the origins and roles of potential circulating inhibitors may result in novel strategies aimed at the prevention or reversal of the life-limiting calcifying vasculopathies seen in CKD patients (12), and a better understanding of the complex mechanisms regulating tissue calcification may have therapeutic potential in reducing the cardiovascular disease-associated morbidity and mortality in patients with renal disease.

2.4.1 Mineral imbalance: the role of calcium and phosphate

Normal serum concentrations of Ca and inorganic P ions are metastable with respect to basic calcium phosphate (BCP; a mixture of octacalcium phosphate, dicalcium phosphate dihydrate and apatite) precipitation, but can support growth of nascent crystals. In ESRD condition, systemic Ca and inorganic P concentrations typically exceed 2.4 and 2.0 mM, respectively (71). Consequently, calcification has traditionally been ascribed to supersaturation and subsequent precipitation of mineral ions. This has led to therapeutic measures to reduce the Ca/P product aimed mostly at reduction of P (70).

More recently, *in vitro* models have been developed to determine the factors specific to CKD that might induce VSMCs calcification. Exposure of VSMCs to media containing elevated levels of calcium or phosphate rapidly induced calcification, with synergistic effects if both ions were elevated (70). Apoptosis accounted for part of the accelerated calcification observed in this model: however, in response to extracellular calcium, viable VSMCs were induced to release vesicles that contained preformed calcium-phosphate apatite accounting for their increased calcification capacity *in vitro* (12, 70). Interestingly, micro- or nano-scale hydroxyapatite and other calcium phosphate crystals may also have the ability to regulate cell phenotype (25, 73, 74). Elevated calcium induces VSMC death and increases matrix vesicle release, whereas calcium and phosphate both increase the mineralization potential of the released matrix vesicles. Figure B offers a complete description of vascular calcification process.

The disturbances in mineral metabolism in CKD possibly have similar effects *in vivo* (75). The heightened state of supersaturation that exists in plasma favors the deposition of mineral in soft tissues, including the blood vessels and cardiac valves, by promoting vesicle release and VSMCs osteogenic differentiation (12).

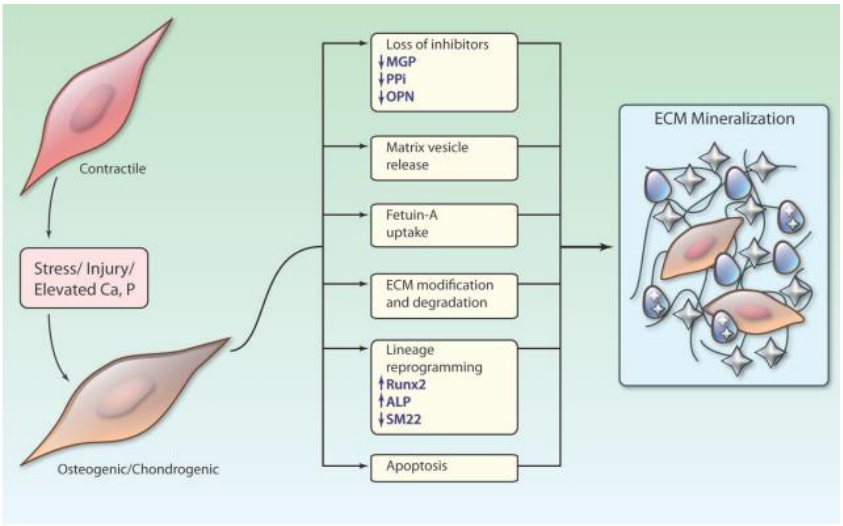

Figure B: Vascular calcification is mediated by vascular smooth muscle cells
Alterations in calcium (Ca) and phosphate (P) levels or vascular insult lead to osteogenic/chondrogenic conversion of VSMCs in the vascular wall. This is associated with dramatic loss of mineralization inhibitors, the production of calcifying matrix vesicles, and extracellular matrix (ECM) degradation. In addition, Ca and P induce VSMC apoptosis and release of apoptotic bodies, which, in turn, form the initial nidus for vascular calcification (76).

2.5 VSMCs: a model system to investigate mechanisms of vascular calcification *in vitro*

Vascular smooth muscle cells represent a validated *in vitro* model to investigate cellular and molecular mechanisms involved in the pathogenesis of vascular calcification (12). They normally reside in the media of blood vessels in a differentiated state and are responsible for regulating vascular tone. They exhibit a contractile phenotype and highly express genes that are required for the maintenance of myofilament structure and function. These genes include α-actin, Smooth Muscle 22α (SM22α) and Smooth Muscle-myosin heavy chain (77). However, VSMC phenotype is characterized by the ability to reversibly enter in a synthetic state of proliferation and production of large amounts of extracellular matrix. Transition into the synthetic state is associated with a loss of smooth muscle cell markers associated with contractility: so, VSMCs can be activated from a quiescent, differentiated state into an actively proliferating and synthesizing phenotype. This phenotypic change is associated with loss of smooth muscle cell markers and can be induced by various stimuli *in vitro*, including various growth factors, injury, or mechanical stress (77). This transition is thought to play a role in the pathogenesis of atherosclerosis and Mönckeberg's Sclerosis, because both are associated with decreased expression of VSMC markers in plaques and areas of calcification (30). VSMCs play a key role in inhibiting calcification in the normal vessel, but features

associated with vascular pathological environments impinge on their normal function, induce damage and cause osteogenic phenotypic change that favors the deposition of mineral (12, 53).

VSMCs are thought to be the predominant cells associated with medial calcification, in contrast to intimal calcification, which also involves lipids and inflammatory cells. A number of studies have shown that VSMCs cultured with high phosphate can undergo calcification *in vitro*, that involves the phenotypic transition to osteoblastic, chondrocytic, and osteocytic cells. This *in vitro* model has been widely used for investigating the cellular and molecular mechanisms responsible for vascular calcification (78).

2.6 The role of phosphorus in the development and progression of vascular calcification

Although much progress has been made in the past five years in understanding the mechanisms leading to accelerated vascular calcification in patients with CKD, it remains unclear how an environment high in phosphate can impinge so significantly on the calcification process (79).

In CKD population, vascular calcification is associated with additional non-traditional factors that may be unique to CKD and thus predispose these patients to early and more accelerated calcification (80). One such factor is serum phosphorus, which has been linked to vascular calcification in several studies and is emerging as a key regulator of calcification in the CKD population (72, 81-83). *In vitro* studies and animal models of vascular calcification suggest that Pi not only participates in hydroxyapatite crystal formation, but, also, directly induces osteogenic gene expression when applied at concentrations similar to those in hyperphosphatemic subjects *in vivo* (84, 85).

Hyperphosphatemia predisposes these patients to early and progressive vascular calcification: it appears to be involved in a number of mechanisms that trigger and promote the progression of vascular calcification, including (1) transition of VSMCs from a contractile to an osteochondrogenic phenotype and mineralization of VSMC matrix through sodium-dependent phosphate cotransporters, (2) induction of VSMC apoptosis, (3) inhibition of monocyte/macrophage differentiation into osteoclast-like cells, (4) elevation of fibroblast growth factor 23 levels, and (5) decreases in Klotho expression.

Whether vascular calcification can be prevented or reversed with strategies aimed at maintaining phosphate homeostasis presently is unknown (83).

2.6.1 Phosphate and osteochondrogenic phenotypic change in VSMCs

To determine whether phosphorus directly affects vascular calcification, several *in vitro* studies have been performed using VSMCs. When VSMCs are exposed to high levels of inorganic phosphate, consistent with levels seen with hyperphosphatemia, calcification is induced in the extracellular matrix surrounding the VSMCs (86). This calcification has features similar to calcification that occurs in bone, including matrix vesicles and bioapatite synthesis (20). Furthermore, phosphate directly induces phenotypic changes in VSMCs, causing them to transform from a contractile phenotype into an osteochondrogenic phenotype. When VSMCs are exposed to elevated phosphate concentrations *in vitro*, there is increased gene transcription of messenger RNAs encoding proteins involved in matrix mineralization and bone formation, such as osteocalcin and Cbfα-1/RUNX2 and simultaneous down-regulation of transcription factors for smooth muscle cells (53, 87). Notably, these changes also occur in human and animal models of calcification (83).

In vitro studies have examined the response of cultured human aortic smooth muscle cells to different levels of extracellular Pi and have demonstrated that cells exposed to Pi levels similar to those seen in uremic patients (> 1.4 mM) showed dose-dependent increases in cell culture calcium deposition. The results of this study also defined the role of elevated phosphate in transforming the vascular phenotype of these cells to an osteogenic phenotype, such that a predisposition for calcification was created. Similarly, in a study of bovine aortic VSMCs, mineralization of VSMCs in culture was associated with the dramatic loss of smooth muscle-specific gene expression (smooth muscle lineage markers SM22 and α-smooth muscle actin) in the presence of an organic phosphate donor, β-glycero phosphate (75). A third study (88) has shown that medial cells from calcified arteries of matrix gla-protein (a calcification inhibitor) null mice express high levels of osteopontin and Cbfα-1, as well as decreased levels of α-smooth muscle actin, when compared with VSMCs from non-calcified wild-type blood vessels. It is interesting that evidences for similar expression patterns in calcified human arteries of patients with calcific uremic arteriolopathy have recently been reported (89). These data support the concept that VSMCs undergo

phenotypic conversion to osteogenic cell type in the presence of hyperphosphatemia in both animals and humans. Similarly in the bone, Cbfα-1 seems to be a key regulatory factor in vascular calcification, being up-regulated by uremic toxins in dialysis patients (90). For example, calcified inferior epigastric arteries from dialysis patients express Cbfα-1/RUNX2 and osteopontin in both the media and intima layers (2). Giachelli et al hypothesized that this phenotypic change in VSMCs may serve to repair or adapt to a mineralizing environment, given the increased expression of several mineral-regulating molecules in the VSMCs (86). The mechanisms responsible for controlling this phosphate-induced phenotypic change currently are not completed elucidated (83). An hypothesis is that the effects of hyperphosphatemia are mediated by a sodium-dependent phosphate co-transporter (NPC), that facilitates entry of Pi into vascular cells (87). This intracellular influx is increased during hyperphosphatemia, as seen in uremic patients, and leads to the accumulation of intracellular phosphate. By pathways that have not yet been fully elucidated, the increased intracellular phosphate serves as a signal for osteogenic gene expression (Cbfα-1 and downstream targets osteopontin and osteocalcin) and as a suppressor of VSMC specific gene expression (smooth muscle lineage markers SM22 and α-smooth muscle actin), resulting in increased secretion of mineral-nucleating molecules (matrix vesicles, calcium-binding proteins, alkaline phosphatase and collagen-rich extracellular matrix). These factors combine to transform the cell to be susceptible to vascular calcification (75, 90).

2.6.2 Phosphate-induced apoptosis in VSMCs

Some studies found a link between apoptosis of VSMCs and vascular calcification: it has been suggested that apoptosis is a key regulator of VSMC calcification (36, 83, 91, 92). Matrix vesicles (MVs) and apoptotic bodies (ABs) appear to have a role in apoptosis-induced vascular calcification: ABs are calcium-enriched membrane-bound vesicles ~ 0.3 to 1.0 μm in diameter released from apoptotic VSMCs, MVs are smaller (30 to 300 nm) and may be remnants of apoptotic cells (91, 93, 94). For example, in advanced carotid artery atherosclerotic plaques, Kockx et al (92) found that the matrix vesicles were derived from VSMCs and contained the pro-apoptotic protein BAX (BCL2-associated X protein).

Evidences support the role of apoptosis in calcification: some studies demonstrated that VSMC apoptosis occurs before the onset of calcification, and that inhibition of apoptosis

leads to a significant decrease in calcification (36). Proudfoot et al. reported that, in a human vascular calcification model, apoptosis occurred before the onset of calcification, and they demonstrated that inhibiting apoptosis also inhibits calcification, and stimulating apoptosis increases calcification. Apoptotic bodies derived from VSMCs may act as nucleating structures for calcium crystal formation (36). In 2008, Shanahan et al. demonstrated that dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis: in the normal vessel wall, VSMCs were morphologically contractile, there was no evidence of extracellular vesicles, and the nuclei showed normal appearance and distribution of heterochromatin (93); however, VSMCs in dialysis vessels showed apoptosis and damage characterized by increased electron density of nuclear heterochromatin, cell shrinkage, and/or vesicle release. In this study, they hypothesize that Ca^{++} accumulation in the vessel begins in response to increased Ca^{++} and P, but protective mechanisms such as adequate mineralization inhibitor levels and extrusion of intracellular Ca^{++} via vesicle release preserves normal VSMC function. In the dialysis milieu, uremic toxins and the continued exposure to high Pi lead to apoptosis. This, in turn, increases local Ca^{++} levels and reduces local levels of VSMC-derived mineralization inhibitors, which potentiates osteo/chondocytic differentiation of smooth muscle cells and the release of pro-calcific vesicles that form a nidus for calcification (36).

However, Giachelli et al (86) found that calcification of VSMCs does not appear to require apoptosis for the initiation of calcification: nonetheless, apoptosis appears to accelerate the calcification process once calcium phosphate crystals are deposited in the matrix. It has been demonstrated that both synthetic and human atherosclerosis-derived calcium phosphate crystals are phagocytosed by VSMCs, resulting in a rapid rise in intracellular calcium concentrations and resultant inflammation and cell death (73, 95). These effects were inhibited by the lysosomal proton pump inhibitor, bafilomycin A1 (73).

How increased phosphorus concentrations result in apoptosis is unclear, but this process may be related to disruptions in normal mitochondrial energy metabolism (96). Mitochondria, in addition to supplying cellular energy, play a central role in the intrinsic apoptotic pathway. Mitochondria-mediated apoptosis involves the release of cytochrome c from the inner membrane space to the cytosol, which in turn triggers the activation of caspase-9 and -3 cascades (97, 98). These apoptotic events are closely linked to

mitochondrial dysfunction, which exhibits changed mitochondrial membrane potential, increased oxidant generation as a result of the perturbation of electron transport chain reaction, and decreased intracellular ATP content because of oxidant-insulted low respiratory activity (99-101). Although the precise mechanisms for mitochondria mediated apoptosis remain to be elucidated, oxidative stress caused by endogenously and exogenously excessive oxidant insults and/or impaired oxidant defenses is generally believed to be key in both mitochondrial dysfunction and cellular apoptosis (102). Mitochondria-targeted antioxidants could inhibit the peroxidation of mitochondrial components including cytochrome c and consequently block apoptosis (103, 104).

Furthermore, recent studies demonstrated that the Pi-induced VSMC apoptosis and subsequent calcification are dependent on the down-regulation of the Gas6/Axl/Akt survival pathway, that inhibits apoptosis and increases survival of VSMCs (35, 105). In fact, Son et al. demonstrated that the expression of Gas6 and Axl is markedly down-regulated in Pi-induced VSMC calcification. For instance, 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) protect VSMCs from Pi-induced calcification by suppressing apoptosis via restoration of Gas6/Axl/Akt survival pathway (35). They examined the role of Gas6-Axl interaction in the processes of apoptosis and calcification: they demonstrated that the addition of recombinant human Gas6 (rhGas6) to human VSMCs *in vitro* significantly inhibited both Pi-induced apoptosis and calcification. Furthermore, addition of Axl-extracellular domain to block the binding of Gas6 to Axl clearly abrogated the inhibitory effect of Gas6 (35). Consistent with a study by Lee et al. (106), the pro-survival phosphorylation of Akt was down-regulated by Pi, and rhGas6 abrogated the decrease. Wortmannin, a specific PI3K inhibitor, abolished the rhGas6-induced phosphorylation of Akt and further eliminated the inhibitory effect of Gas6 on Pi-induced apoptosis and calcification (106). These findings indicate that the preventive effect of Gas6 on Pi-induced apoptosis and calcification is mediated by the PI3K-Akt pathway. It is well established that PI3K-Akt affects cell death through the Bcl2 family, critical regulators of apoptosis (106). In VSMC calcification, Pi inactivated Bcl2 and activated Bad, followed by caspase-3 activation, finally leading to apoptosis.

There could be a close interplay between phosphate-induced VSMC phenotype change and apoptosis, as proposed recently by Shanahan's group (107): VSMCs that adapt to the hostile

conditions by undergoing lineage reprogramming from contractile to synthetic bone forming phenotype are able to secrete matrix vesicles, thus avoiding calcium overload. In contrast, VSMCs that fail to differentiate succumb to apoptosis, which also results in budding of vesicles and matrix mineralization. Both pathways lead to extracellular calcium phosphate deposition with increased risk of apoptosis of surviving VSMCs (95).

2.6.3 Phosphate and autophagy in VSMCs

Autophagy seems to be involved in VSMC vascular mineralization. The term 'autophagic cell death' has been widely employed to indicate a type of cell death that is accompanied by massive vacuolization of the cytoplasm (108). However, the relationship between autophagy and cell death remains controversial. *In vivo* studies on Drosophila melanogaster have provided evidences that cell death can be, at least partially, executed through autophagy (109-112) and, consistent with these results, the knockout/knockdown of essential autophagy genes has been shown to protect cultured mammalian cells from some lethal inducers (113). Nonetheless, more frequently, pharmacological and genetic inhibition of autophagy doesn't prevent cell death, and, rather, accelerates it (114, 115). This evidences suggest that, although cell death can occur together with autophagy, the latter likely represents a pro-survival mechanism activated by dying cells in the attempt to cope with stress (114, 115).

Autophagy is a dynamic and highly regulated process of self-digestion. It is a highly conserved cellular process responsible for removal or recycling of long-lived proteins and organelles, and it provides cells with an alternative source of nutrients from the reuse of cellular proteins and organelles. This lysosomal degradation pathway is essential for cell survival, differentiation and development, as well as the cellular response to stress. Limited autophagy in response to nutrient starvation has a survival function, and specific removal of damaged mitochondria by autophagy can prevent the activation of apoptotic pathways.

Dai et al. demonstrated that, although the inhibition of autophagy reduced phosphate-induced VSMC apoptosis, it caused an increment in calcium deposition in both animal (bovine and rat VSMCs) and human aortic cells. Moreover, their results shown that the induction of autophagy is correlated with a decrease of calcification in bovine VSMCs and rat aortic rings. So, autophagy could be an endogenous protective mechanism counteracting

phosphate-induced vascular calcification not acting by the decrease of VSMC apoptosis, but acting by the reduction of matrix vesicle release (116).

2.7 Potential role of the sodium-dependent phosphate cotransporter Pit-1 in vascular calcification

Phosphate transport into cells is primarily mediated by sodium-dependent phosphate cotransporters, and three types of cotransporters have been identified based on structure, tissues expression and biochemical characteristics (18, 117, 118) The type I and type II sodium-dependent phosphate cotransporters are primarily expressed in kidney and intestinal epithelium, and their functions are important for the maintenance of phosphate homeostasis in the body (117, 118). The type III sodium-dependent phosphate cotransporters, Pit-1 and Pit-2, were originally identified as cell surface receptors for the gibbon ape leukemia virus (Glvr-1) and the amphotropic murine retrovirus (Ram-1), respectively. Type III members are widely distributed in kidney, liver, lung, heart, brain, osteoblasts, chondrocytes and VSMCs (84, 87).

A functional sodium-dependent phosphate transport system has been characterized in VSMCs (84, 87). RT-PCR revealed expression of type III sodium-dependent phosphate cotransporters, Pit-1 and Pit-2, while no transcripts for type I and type II sodium-dependent phosphate cotransporters were detected. Real-time PCR indicated that Pit-1 mRNA levels were higher than Pit-2 (84). Treatment of VSMCs with a competitive inhibitor of sodium-dependent phosphate cotransporters, phosphonoformic acid (PFA), caused a dose-dependent inhibition of phosphate uptake, calcification, and osteochondrogenic phenotype in VSMCs (87, 119). These results suggest that phosphate transporter activity is necessary for mineralization as well as osteochondrogenic transition in human VSMCs.

Since PFA has lower affinity for type III than for type II receptors (120), the requirement of phosphate uptake for VSMC calcification was further examined using VSMCs that were stably transduced with Pit-1 specific small hairpin RNA (shRNA) (84). Pit-1 shRNA expressing cells had reduced mRNA and protein levels of Pit-1 as detected by Northern and Western blots, respectively. Sodium-dependent phosphate uptake in the cells was reduced compared to that in control cells. After incubation with elevated phosphate for 7, 10 or 14 days, there was substantially reduced calcification in Pit-1 knockdown cells compared to control cells. Of

interest, restoration of phosphate uptake in Pit-1 knockdown cells by over-expression of mouse Pit-1 rescued elevated phosphate-induced mineralization. Similar to PFA, inhibition of phosphate uptake by Pit-1 shRNA blocked the expression of phosphate-induced osteogenic differentiation markers, Runx2 and osteopontin. These studies indicated that sodium dependent phosphate cotransporters, in particular Pit-1, might be a major mechanism for controlling vascular calcification and VSMC phenotypic state. Taken together, these results demonstrate that phosphate transport via Pit-1 is required for calcification in cultured VSMCs.

Recent studies have supported a role for increased phosphate uptake via Pit-1 in vascular calcification *in vivo*. Mizobuchi *et al* showed that mRNA levels of Pit-1 and Runx2 were increased in calcified aorta of uremic rats with severe hyperparathyroidism, while no increase was observed in non-calcified aorta of control animals (121). Likewise, *LDLR*−/− mice fed with a high-fat diet showed elevated levels of serum tumor necrosis factor α (TNF-α) and Pit-1 in calcified aortic calcification. *In vitro*, several factors that have been shown to induce vascular calcification also induce Pit-1 in VSMCs. Long-term treatment of human VSMCs with elevated calcium levels leads to increased Pit-1 mRNA levels, phosphate uptake and calcification (122). Likewise, PDGF promotes calcification in cultured SMCs and strongly induces Pit-1 expression (90). In addition, bone morphogenetic protein 2 (BMP-2), a potent osteogenic protein, has been shown to promote vascular calcification (24, 41, 123) increasing phosphate uptake in a time-and dose-dependent manner (124). Interestingly, BMP-2 also promoted mineralization and upregulated Pit-1 expression in osteoblasts, and BMP2-enhanced mineralization in these cells was abrogated by Pit-1 siRNA (125). Finally, transglutaminase (TG) appears to be an important regulator of Pit-1 endogenous expression, since Pit1 levels were observed in *TG+/+* SMCs but absent in *TG*−/− SMCs in the presence or absence of elevated phosphate (126).

Thus, it is likely that phosphate transport via Pit-1 is a common requirement for cell-mediated biomineralization (18).

2.8 Calcium phosphate nanocrystals promote VSMC osteogenic gene expression

Despite a plethora of studies addressing multiple aspects of P-induced calcification, results from different models and systems have often provided conflicting data, casting doubt on

the notion that uptake of P alone, acting via these signaling pathways, is all that is required to promote calcification. Also the presence of nanocrystalline Ca/P could lead to a pathological positive-feedback loop causing cell death, inflammation, phenotypic change, matrix degradation and calcification (79).

The size of crystals is important in determining their effects on target cells (73): Ca/P crystals in the range of 1 -2 μm or less in diameter were endocytosed, caused toxic effects in VSMCs and induced the release of proinflammatory cytokines from macrophages, whereas crystals with a diameter greater than 20 μm are mostly inert. This is possibly due to lack of cellular uptake of these larger particles, which may be dissolved by alternate extracellular mechanisms that do not induce the same phenotypic effects as nanocrystal uptake (127). This implies that calcification is damaging in its earliest stages, prior to crystal growth or aggregation, making this initial stage the most important target for prevention.

The study by Sage et al. highlights an important and novel role for calcium phosphate nanocrystals produced in a high-phosphate environment. Importantly, the presence of these crystals led to an increased expression of the osteogenic proteins BMP-2 and osteopontin in VSMCs, proteins that were not upregulated by high P alone (25).

VSMCs undergo both apoptosis and phenotypic change at sites of calcification. In combination, all these factors appear to be required for the deposition of Ca/ P nanocrystals in the vessel wall.

2.9 Therapeutic strategies for vascular calcification treatment

Relatively new pharmacological agents are used either alone, or in combination, to minimize hyperphosphatemia and hyperparathyroidism associated complications to improve morbidity and mortality of CKD patients (128).

With time, new formulations of phosphorus binders have been developed to minimize side effects. For instance, once widely used aluminum-based phosphorus binders can induce anemia, myopathies, dementia and bone anomalies. Calcium-based binders, though somewhat effective, are also linked to vascular calcification in patients undergoing hemodialysis treatment (128). Recently, sevelamer carbonate, a non-calcium and non-aluminum-based binder have been shown to reduce vascular injury better than calcium-based binders (129, 130).

Another phosphate-binding agent used to reduce the gastrointestinal absorption of phosphate and ameliorate vascular calcification in advanced CKD is lanthanum carbonate (131). It has been extensively investigated in preclinical and clinical studies, demonstrating good efficacy in reducing serum phosphate levels, without major side effects, over 6 years of treatment (132).

Additionally, cationic lanthanum (La^{+++}), primarily based on its similarity to Ca^{++}, can activate Calcim Sensing Receptor (CaSR), that is expressed in all the organs and cells that maintain systemic Ca^{++} (131).

Even calcimimetics, such as cinacalcet and calindol, are positive allosteric modulators of the CaSR, interacting with the membrane-spanning domain of the receptor to induce a conformational change that enhances signal transduction. This change in conformation leads to increased sensitivity to extracellular calcium and subsequently to a decrease in circulating PTH levels. Besides changing the structural conformation, calcimimetics can also enhance CaSR function by increasing its expression (133).

2.9.1 Calcium sensing receptor (CaSR) and vascular calcification

The consequence of the activation of the CaR and the cascade of intracellular signals the reduction in serum calcium concentration through the increase of urinary calcium excretion, the decrease of bone turnover, and the decrease of intestinal absorption of calcium.

Noteworthy, studies have demonstrates that the expression of CaSR is reduced in human calcified arteries and in mineralized VSMCs, suggesting that it could play a preventive role in vascular mineralization when expressed normally, and that a functional CaSR is necessary to maintain a VMSC phenotype.

Additional, *in vitro* studies have also demonstrated that calcimimetics decrease calcification of human smooth muscle cells when cultured in high-phosphate medium; this inhibitory effect is abolished by the transfection in these cells of a silencing CaSR RNA (SiRNA-CaR), suggesting that the direct activation of the CaSR in vascular cells may slowdown the calcification process. Calcimimetics may also prevent vascular calcification by modulating the expression of several genes involved in bone mineralization: they up-regulate MGP and down-regulate PiT1. Moreover, they are able to decrease the vascular expression of bone morphogenic protein-2, obstructing the osteoblastic transdifferentiation of VSMCs. The

activation of the CaSR by calcimimetics could also favor the regression of vascular calcification through the stimulation of macrophages or phagocytic cells adjacent to calcified aortic lesions as found in an *in vivo* model of 5/6th nephrectomized rats.

Altogether, the results of these *in vitro* and *in vivo* studies point toward a direct implication of the CaSR in the mechanisms of vascular function and mineralization (133).

3. AIM OF THE STUDY

Since more than 90% of CKD patients dies for cardiovascular events and the main uremic toxin involved in cardiovascular calcification is high phosphorous, we decided to develop an *in vitro* model to elucidate the molecular and cellular mechanisms involved in the pathogenesis of vascular calcification.

In particular, we studied this pathological process in VSMCs challenged for 7-15 days with high Pi (inorganic phosphorous). Our model has the purpose to reproduce *in vitro* the same pathological process that occurs in CKD patients *in vivo*.

Known the ability of VSMCs to remodel their phenotypic characteristics in response to environmental changes and known the primary role of high phosphorous on vascular calcification and on osteoblastic differentiation of VSMCs, the main purpose of our *in vitro* studies was to develop potential strategies that allow VSMCs to contrast the progression of high Pi-induced calcium deposition.

In the first part of these studies, we tried to better elucidate the role of the pleiotropic protein osteonectin (SPARC) in the process of vascular calcification, because, currently, the studies about the modulation of this protein expression in vascular calcification are few and controversial. Therefore, our purpose was to investigate its role in our model of vascular calcification *in vitro* and *ex-vivo*.

In the second part of the project, we investigated the potential effect of repeated and short time suspensions of high Pi treatment (process that we called “Wash Out”) on the progression of calcium deposition *in vitro*, trying to reproduce the same temporal decrease of Pi levels that occurs in CKD patients treated with haemodialysis three times weekly. Moreover, our purpose was to investigate which are the high Pi induced molecular pathways responsible for the pathological calcium deposition in the vasculature that could be potentially modulated by the “Wash Out” treatment.

The third part of the project is a pharmacological study that has the intention to investigate the mechanism of action of Lanthanum Chloride ($LaCl_3$), Gadolinium Chloride ($GdCl_3$) and

Calindol on the delay of the progression of high Pi-induced calcium deposition and on the osteoblastic transformation of VSMCs.

4. MATERIALS

DMEM (high glucose, [4,5 g/l]), NaCl, FBS and BCA protein assay kit (Pierce) were purchased from Euroclone (Milan, Italy); Na_3PO_4, $MgSO_4$, NaH_2PO_4, KH_2PO_4 and KCl were from Carlo Erba (Milan, Italy); $CaCl_2$ was from BDH (Bristol, England); calcium kit was from Pokler Italia (Salerno, Italy); primary antibody for α-actin (A2547) and anti-mouse secondary antibody (A9917) were from Sigma (St.Louis, MO, USA); primary antibody for SM22α (ab10135) and anti-goat secondary antibody (ab6741) were from Abcam (Cambrige, UK); primary antibody for Axl (sc-1097) and Calindol hydrochloride were from Santa Cruz (Heidelberg, Germany); primary antibody for LC3-II was from Cell Signalling (Danvers, USA) and anti-rabbit secondary antibody was from GeneTex (Irvine, USA); primary antibody for osteonectin (N50) was from Biodesign International (Saco, ME, USA); primary antibody for Ki67 (mib1) was from Dako (Glostrup, Denmark); Hepes Buffer Solution, PVDF membrane, Taqman gene assay for Cbfα1/RUNX2 (Rn_01512298), BMP-2 (Rn_00567818), MGP (Rn_00563463) and α-actin (Rn_00667869) and all reagents for gene expression assays were from Invitrogen/Applied Biosystem (Milan, Italy); PFA was from Fluka; cell-death detection ELISA plus Kit was from Roche. Unless otherwise mentioned, all the other reagents were obtained from Sigma (St.Louis, MO, USA).

5. METHODS

5.1 Induction of calcification in VSMC *in vitro* model

Male Sprague-Dawley rats were killed by intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg), and this protocol fully complied with recommendations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Rat VSMCs were obtained by enzymatic digestion and they were routinely sub-cultured in growth medium (DMEM containing 10% FBS supplemented with 100U/ml penicillin, 0.1mg/ml streptomycin). At 80% confluence, cells were switched to calcification medium (DMEM containing 12% or 15% FBS supplemented with 100U/ml penicillin, 0.1mg/ml streptomycin, 10mM sodium pyruvate and the pro-calcific factors 5mM Pi and 50µg/ml ascorbic acid) for up to 15 days.

Medium was replaced every 2 or 3 days.

For time-course experiments, the first day of culture in calcification medium was defined as day 0.

- In “Wash Out” experiments, we define “Wash Out” treatment the temporary suspension of high Pi every time medium was replaced: in particular, 5mM Pi treatment was suspended for 2 or 4 hours every time medium was replaced to be, then, added again after the “Wash Out” period.
- In the pharmachological study, $LaCl_3$, $GdCl_3$ and Calindol were added 2 hours before the addiction of high Pi every time medium was replaced.

Cells were used between the sixth and eight passage, and calcium deposition was quantified using two alternative approaches, o-cresophtalein method and Alizarin Red S staining followed by $HClO_4$ destaining and spettrophotometrical determination.
Ca^{++} deposition of the cell layer was normalized to protein content and expressed as µg Ca^{++}/mg protein or as absorbance (OD)/mg protein.

5.2 Quantification of calcium deposition in VSMCs

For the quantification of calcium deposition, we utilized two alternative approaches: o-cresophtalein method and Alizarin Red S staining followed by $HClO_4$ destaining.
For the former method, cells were decalcified by incubation for 24 hours with 0.6 M HCl, and Ca^{++} content in HCl supernatants was determined colorimetrically at 575nm wavelength by the o-cresophthalein complexone method.
For the latter, extracellular calcium deposits were stained with Alizarin Red S solution for 30 minutes to be, then, destained for 24 hours with 5% perchloric acid, and Ca^{++} content in $HClO_4$ supernatants was determined colorimetrically at 450nm wavelength.
After decalcification, cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% SDS. Protein content was quantified by BCA protein assay kit (Pierce), and Ca^{++} deposition of the cell layer was normalized to protein content and expressed as µg Ca^{++}/mg protein or as absorbance (OD)/mg protein.

5.3 Alizarin Red, Von Kossa and Immunohistochemistry

To visualize calcium deposition, cells were grown on plastic supports and, at the end of the experiment, they were fixed with 70% EtOH and stained with 1mg/ml Alizarin Red S solution for 30 minutes. Cells were rinsed and Ca^{++} deposition was photographed.

Besides rat VSMCs, we analyzed human arteries *ex vivo* by isolating, during autoptic examination, aortic walls from a fetus at 20th gestation weeks, a 35 years old young adult with hypertension, but without macroscopically evident arteries injury and two over 65 years old adults with and without calcified atheromatous plaques.

For Von Kossa and immunohistochemistry analysis, cell cultures and arteries were fixed in formalin and paraffin embedded, and 3 µm sections were stained.

Von Kossa tissue staining was performed incubating slides with a 5% silver nitrate solution reduced under UV lamp for 1 hour, then, after 2-3 minutes in 5% sodium thiosulfate solution, were counterstained with safranine. Calcium deposits were visualized as black metallic silver granules. The deposition of calcium salts by Von Kossa staining was evaluated as the presence of fine granules or large calcific deposits. Cultured cells evaluation focused on the presence or absence of the same, on their arrangement (small or large aggregates), and on the morphology (round or spindle).

Alizarin Red and Von Kossa expression were evaluated with a semi quantitative method, using a score system (0-4) related to the stained area (0 < 10%; 1 = 11-25%; 2 = 26-50%; 3 = 51-75%; 4 > 75%).

The evaluation of osteonectin protein was performed using monoclonal antibody to osteonectin (1:30,000), while cell proliferation with monoclonal antibody to Ki67 (1:100) incubated 1 hour at RT. For immunohistochemistry stain, Biogenex i6000 Automated Staining System (Biogenex, freemont, CA, USA) was used. The reaction was detected by Novolink Max polymer detection system (Novocastra Laboratories L.T.D., Leica Microsystem), following the manufacturer's instructions, using as chromogen Diaminobenzidine (DAB), incubated for 8 minutes at RT.

Osteonectin expression was assessed with semi-quantitative methods:

1) Percentage of <u>positive cells</u> was related to the stained area: 0 ≤ 10%; 1 = 11-25%; 2 = 26-50%; 3 = 51-75%; 4 > 75%.

2) Staining intensity was scored: 0 = absence of positive cells; 1 = positive cells with weak intensity; 2 = positive cells with intermediate intensity and 3 = positive cells with high intensity.
3) Cellular distribution was scored: 1 = para-nuclear; 2 = cytoplasmic and membranous diffuse.

   Proliferation index (Ki-67) was determined counting positive cells on 100 VSMC at high magnification (40x). Ki-67 was reported as percentage with a semi-quantitative score: 0 = 0; 1 ≤ 10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%. When 100 cells were not available, stained cells on total available cells were related to 100.

5.4 Electron microscopy in VSMCs

Samples of cells were scraped from culture flask, fixed in 2.5% glutaraldehyde in 0.13M phosphate buffer pH 7.2-7.4 for 2 hours, post-fixed in 1% osmium tetroxide, dehydrated through graded ethanol and propylen oxide and embedded in epoxy resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate, to be observed in a Jeol JEM 1010 transmission electron microscope (Jeol, Tokyo, Japan).

5.5 RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using PureLink RNA Mini Kit according to the manufacturer's instructions. The concentration of RNA was measured using a UV spectrophotometer. Reverse transcription was performed with high capacity RNA-to-cDNA kit. All TaqMan PCR was performed using a StepOne Real-Time PCR System. Each reaction mixture (20µl) contained 10µl of 2X TaqMan Universal PCR Master Mix, 1µl of Taqman gene assay, 9µl of cDNA sample [(4ng (MGP), 50ng (Cbfα1/RUNX2), or 100ng (BMP-2)] and water. The thermal cycling conditions comprised the initial steps at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Amplification of the target genes was normalized to simultaneous amplification of an internal housekeeping gene, β-actin, and calibrated to a low expressing, normalized, target sample.

5.6 Western Blot

Rat VSMCs were harvested in ice-cold homogenization buffer (50mM Tris pH 8 with 0.5% IGEPAL CA-630, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM benzamidine HCl, 1mM

sodium fluoride (NaF), 10mM β-glycerophosphate and complete protease inhibitor), freeze-thawed 2X and sonicated 5 x 20 s at 40% power. Samples were then centrifuged at 13,000 g for 15 minutes at 4°C, and protein concentration was measured. Denatured samples (5, 1 or 50 µg total protein for α-actin, SM22α and Axl and LC3-II, respectively) were separated by electrophoresis on a 12% (α-actin, SM22α and LC3-II) or 7.5% (Axl) SDS-polyacrylamide gel and, then, were transferred to PVDF membrane. Membranes were incubated 1 hour with 1:7,500 (α-actin) or 1:2,500 (SM22α), or O/N with 1:200 (Axl) or 1:300 (LC3-II) primary antibodies, followed by 1 hour incubation with 1:40000 (α-actin) peroxidase-conjugated anti-mouse, 1:5,000 (SM22α) or 1:10,000 (Axl) peroxidase-conjugated anti-goat or 1:20,000 (LC3-II) peroxidase-conjugated anti-rabbit secondary antibodies. Protein bands were visualized using an ECL detection kit, and area intensity was measured.

For the analysis of LC3-II marker, some cells were pre-incubated O/N at 37°C with 25µM chloroquine the day before sample harvest.

5.7 Detection of apoptosis

Cytoplasmic histone-associated DNA fragments were detected with a cell-death detection ELISA plus kit as a quantitative index of apoptosis. Briefly, after VSMCs were scraped in lysis buffer and sonicated 5 x 20 s at 40% power, samples were centrifuged at 200 x g for 10 min at RT, and 20µL of supernatant (cytoplasmic fraction) was used for the assay. Following the addition of substrate, colorimetric change was determined by the absorbance value measured at 405nm.

5.8 Short time Phosphonoformic Acid (PFA) treatment in the "Wash Out" model

VSMCs were exposed for 4 hours to 1mM phosphonoformic acid (PFA) in presence of 5mM Pi every time medium was replaced. In the attempt to limit PFA effect on the 4 hours treatment, VSMCs were washed with PBS after this period of incubation, and calcification medium with 5mM Pi was added, again. Calcium deposition was quantified using Alizarin Red S staining followed by $HClO_4$ destaining and spettrophotometrical determination. Absorbance values were normalized by protein content.

5.9 Strategies to study the contribution of active and passive components on vascular calcification in the "Wash Out" model

5.9.1 Short time "Wash Out" samples exposition to "choline-saline solution"

VSMCs were exposed to "choline-saline solution" (1M Hepes Buffer Solution, 25mM D-glucose, 140 mM Choline-Cl, 0.8 mM $MgSO_4$, 0.9 mM KH_2PO_4, 5.36 mM KCl, 1.8 mM $CaCl_2$) or they were treated with the same saline solution with Na^+ instead of choline ("Na^+-saline solution", with 140mM NaCl and 0.9mM NaH_2PO_4 rather than Choline-Cl and KH_2PO_4) in presence of 5mM Pi for 2 hours every time medium was replaced.

Calcium deposition was quantified using Alizarin Red S staining followed by $HClO_4$ destaining and spettrophotometrical determination. Absorbance values were normalized by protein content.

5.9.2 Short time targeted PFA treatment in "Wash Out" samples

Calcification medium cell-free was pre-incubated with 5mM Pi, at 37°C O/N the day before the replacement of medium to allow the precipitation of hydroxyapatite crystals in absence of cells. VSMCs were exposed to this pre-incubated medium in the presence of 1mM PFA for 4 hours every time medium was replaced. Then, cells were washed with PBS and calcification medium was added, again, in presence of 5mM Pi.

Calcium deposition was quantified using Alizarin Red S staining followed by $HClO_4$ destaining and spettrophotometrical determination. Absorbance values were normalized by protein content.

5.9.3 Short time free Pi and calcium-phosphate crystals treatment in "Wash Out" samples

Calcification medium cell-free was pre-incubated with 5mM Pi, at 37°C O/N the day before the replacement of medium to allow the precipitation of hydroxyapatite crystals in absence of cells. Medium was repeatedly centrifuged at 16,000 x g for 5 min at RT in order to allow the isolation of hydroxyapatite crystals (precipitate) from free Pi (supernatant). VSMCs were exposed to hydroxyapatite crystals or free Pi for 4 hours every time medium was replaced, then they were washed with PBS, and calcification medium was added, again, in presence of 5mM Pi.

Calcium deposition was quantified using Alizarin Red S staining followed by $HClO_4$ destaining and spettrophotometrical determination. Absorbance values were normalized by protein content.

5.10 Statistical analysis

Results were expressed as mean ± SEM. Each experiment was performed at least three times at least in triplicate. Differences between groups were analyzed by one-way ANOVA and are considered statistically significant when p value < 0.05.

6. RESULTS

6.1 MODULATION OF OSTEONECTIN EXPRESSION IN VASCULAR CALCIFICATION PATHOGENESIS

6.1.1 Effect of high Pi on VSMCs

VSMCs challenged with 5mM Pi for 7 days calcify. To demonstrate the ability of these cells to deposit calcium, we decided to investigate ultra-structural changes high Pi induced using electron microscoscopy. In high Pi treated VSMCs there are calcified mitochondria, numerous electron dense calcium deposits, mainly in the extracellular matrix near plasma membrane, and there is an extracellular vesicles trafficking (Fig. 1B, C). On the contrary, no calcium deposits and no ultra-structural changes were seen in control samples (Fig 1A).

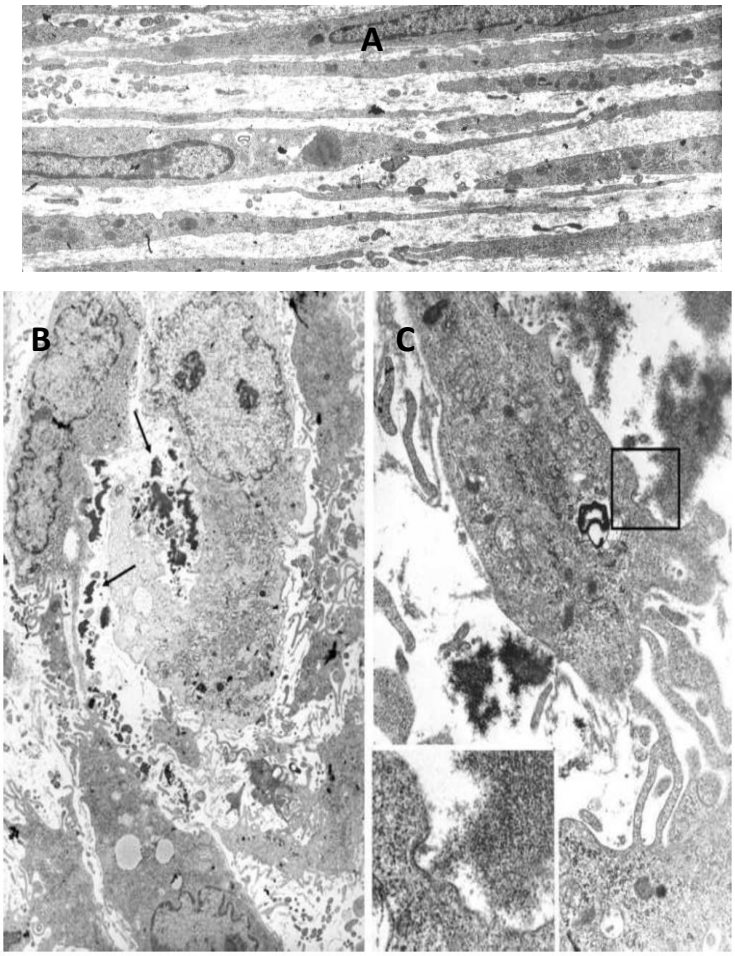

Figure 1: Effects of high phosphate on VSMCs showed by electron microscopy
A: Control VSMCs**; B-C:** 5mMPi.

A: Ultra-structural integrity of normal cells, that have the characteristic morphology of VSMCs. 2,500x original magnification **B**: Electron micrograph showing calcium electron dense deposits in extracellular matrix between neighboring cells (arrows). 2,500x original magnification; **B**: Particular of a cytoplasmic process with calcium deposits close to cell membrane. 10,000x original magnification. **Inset**: Higher magnification of the boxed area showing the remnant of an exocytosis vesicle on membrane near a calcium deposit.

6.1.2 Effect of high Pi on the modulation of VSMC Osteonectin expression

In order to elucidate the potential role of osteonectin in vascular calcification, we evaluated high Pi-induced osteonectin expression time-course in our *in vitro* model from a histological point of view analysing three different parameters: percentage of positive cells, staining intensity, and cellular distribution of the staining. In the time course, three different points have been evaluated: 2, 4, and 7 days of high-Pi treatment.

In controls, at every day tested, osteonectin was present in few VSMCs (score 0), with a faint staining (score 0-1), in paranuclear position (score 1), specifically at 7 days (Fig. 2A).

Under calcification stimuli, after 4 days of challenge, all the parameters in osteonectin staining did not increase significantly (score 1 in expression, intensity, and subcellular position) (Fig. 2B). On the contrary, at 7 days large amount of cells express diffusely osteonectin (score 4) in the cytoplasm (score 2), with stronger intensity (score 3) (Fig 2C). At day 2, osteonectin staining pattern is not different from control cells, and, after 10 days of high-Pi challenge, there are not differences compared to day 7, with a dispersion of osteonectin from cells toward cellular matrix due to the lost of cells vitality *(data not shown)*.

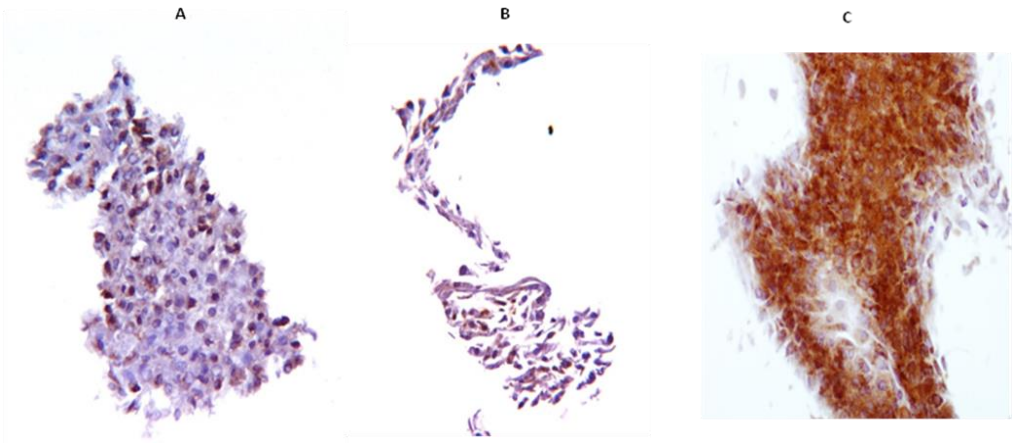

Figure 2: Time course of osteonectin protein expression in high Pi condition
VSMCs were cultured in calcification medium with 5mM Pi, and osteonectin protein expression was measured by immunohistochemistry. Time course shows that 7 days after high Pi challenge there is a significant increase in osteonectin protein levels. **A**: Control VSMCs: osteonectin staining was present in few cells (score 0), with a

faint staining (score 0-1), in paranuclear position (score 1); **B**: 5mM Pi, day 4: osteonectin staining was present in some cells (score 1), with a faint staining (score 1), in paranuclear position (score 1); **C**: 5mM Pi, day 7: large amount of cells express diffusely osteonectin (score 4), with strong intensity (score 3), in the cytoplasm (score 2). Magnification x20. Representative result of one of three different experiments.

To clarify the relationship between osteonectin and vascular calcification, we next analyzed calcium deposition time course with Von Kossa staining, that shows a pattern of expression according with osteonectin.

Control cells do not accumulate calcium deposits (score 0) (Fig. 3A), whereas in VSMCs challenged with high Pi for 4 days there are some calcified deposits (score 1-2) (Fig. 3B), that become more and more apparent and consistent after 7 days of culture in calcification medium (score 2) (Fig. 3C), according to the expression of osteonectin. Started to day 10, calcification was more pronounced also due to cell death *(data not shown)*.

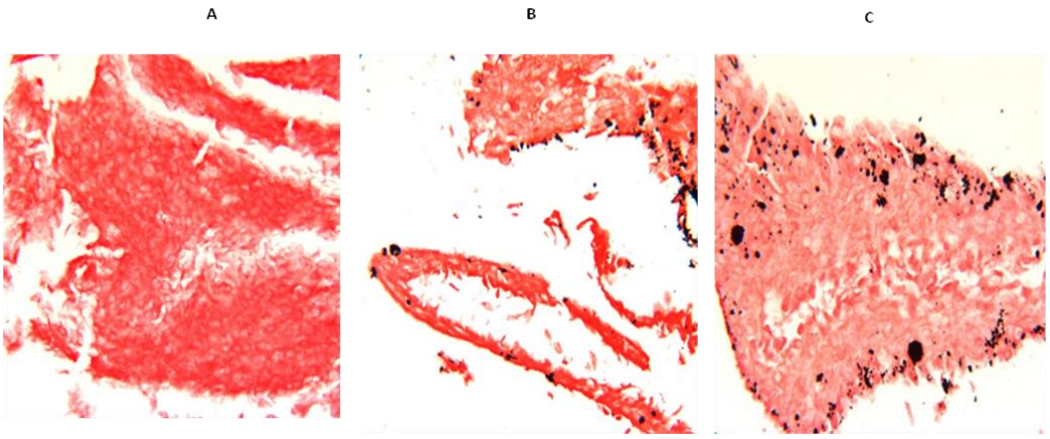

Figure 3: Time course of calcium deposition in high Pi condition
VSMCs were cultured in calcification medium with 5mM Pi, and calcium deposition was measured by Von Kossa staining. Time course shows that 7 days after high Pi challenge there is a significant increase in calcium deposition. **A**: Control VSMCs: no calcium deposits were present in control cells (score 0); **B**: 5mM Pi, day 4: black stained calcium deposits were diffusely present between the cells (score 1-2). **C**: 5mM Pi, day 7: large black stained calcium deposits were massively present between the cells (score 2).
Magnification x20. Representative result of one of three different experiments.

6.1.3 Effect of high Pi on the modulation of VSMC Ki67 expression

Since osteonectin is a protein produced in tissue that undergo remodeling and differentiation, in order to understand whether its expression correlated with cell proliferation in our model, we investigated, with an immunohistochemistry assay, the time course expression of Ki67, a nuclear marker of cellular proliferation.

In controls, at every time point tested, Ki-67 was present in very few cells (score 0) (Fig. 4A). After 4 days of high Pi challenge, the number of nuclei that stain for Ki-67 increased (score 2) (Fig. 4B), decreasing after 7 days (score 0) (Fig. 4C).

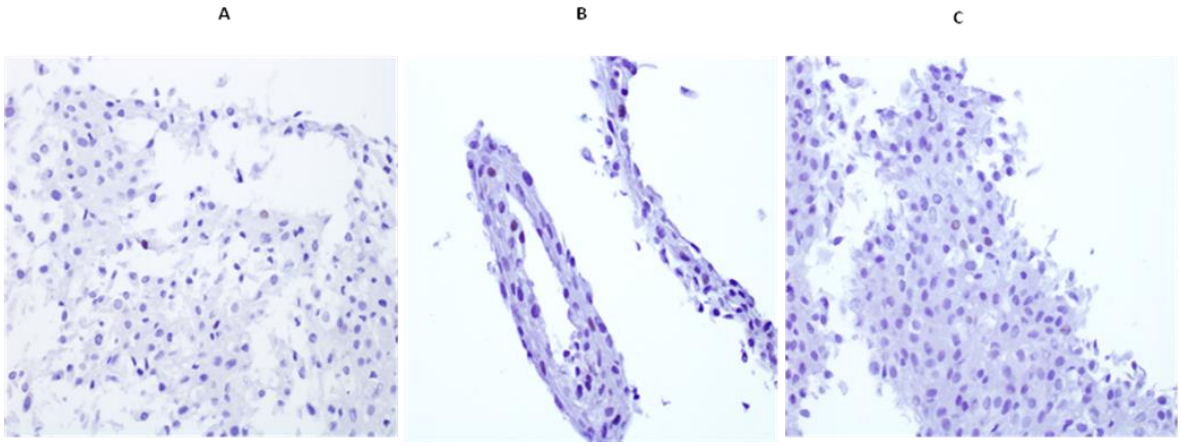

Figure 4: Time course of Ki-67 protein expression in high Pi condition
VSMCs were cultured in calcification medium with 5mM Pi, and Ki-67 protein expression was measured by immunohistochemistry. Time course shows that 4 days after high Pi challenge there is a significant increase in Ki-67 protein levels. **A**: Control VSMCs: in control condition few nuclei stain for Ki-67; **B**: 5mM Pi, day 4: some nuclei stain for Ki-67 (score 2); **C**: 5mM Pi, day 7: Ki-67 staining decrease at control level.
Magnification x20. Representative result of one of three different experiments.

6.1.4 Effect of high Pi on the modulation of VSMC Cbfα1/RUNX-2 expression

As in our model osteonectin expression was not strictly related to proliferation, we tried to evaluate osteonectin association with the expression of the master gene involved in osteoblast differentiation, Cbfα1/RUNX-2, to elucidate if the time of the maximal VSMC transformation correlated with osteonectin expression. Cbfα1/RUNX2 is a transcription factor strongly expressed during the transition of VSMCs towards the osteoblastic-like phenotype when they are exposed to a pro-calcific environment. We investigated, with a real time PCR assay, the time course expression of Cbfα1/RUNX-2 in VSMCs challenged with 5mM Pi up to day 9.

For Cbfα1/RUNX-2, after 7 days of incubation, the challenge with 5mM Pi results in a peak with a 4.44 ± 0.74-fold increase in messenger RNA (mRNA) compared to the relative expression in normal VSMCs (t_0) (*$p < 0.01$) (Fig. 5).

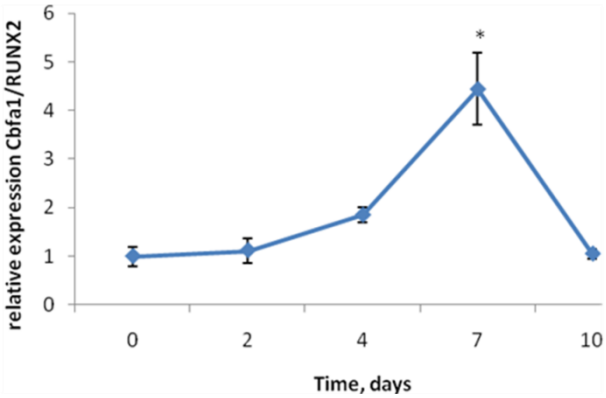

Figure 5: Time course of Cbfα1/RUNX-2 mRNA expression in high Pi condition
VSMCs were cultured in calcification medium with 5mM Pi, and Cbfα1/RUNX2 mRNA expression was measured by RT-PCR. Time course shows that 7 days after high Pi challenge there is a significant increase in Cbfα1/RUNX2 mRNA levels compared to the relative expression in normal VSMCs (t_0).
Data were presented as mean ± SE of three different experiments (*p < 0.01).

6.1.5 Effect of Ascorbic Acid on the modulation of VSMC Osteonectin expression

In order to elucidate the potential role of osteonectin in VSMC calcification, we tried to associate its expression with a factor that we previously demonstrated to be involved in the promotion of the calcification process (134): ascorbic acid (AA).

Figure 6 demonstrates the pro-calcifying effect of AA: when removed from the calcification medium, the high Pi-induced calcification in absence of AA at day 7 decreases compared to the high Pi-induced calcification in presence of AA (32.5 ± 4.7 5mM Pi with AA vs 12.7 ± 0.8 5mM Pi without AA; µg Ca^{++}/mg protein; *p < 0.01).

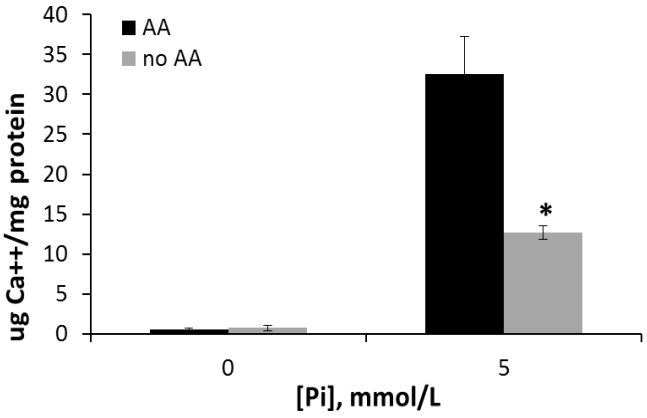

Figure 6: Effect of ascorbic acid on calcium deposition

VSMCs were cultured in calcification medium with 5mM Pi in presence (black bars) or in absence (gray bars) of AA for 7 days. Ca^{++} deposition was measured and normalized by cellular protein content. Removing AA from the calcification medium results in a significant decrease of Ca^{++} deposition.
Data are presented as mean ± SE (*p < 0.01).

Therefore, we studied osteonectin expression modulation by the presence or absence of AA and we chose to analyze its expression at day 7, the same time point of osteonectin expression peak.

Results show that, removing AA from the calcification medium results in a decrease of osteonectin expression, with less cells that diffusely expressed it (score 4 *vs* 3; with or without AA) and with a less strong intensity (score 3 *vs* 2; with or without AA), but with the same cytoplasmic subcellular localization (Fig. 7A-B).

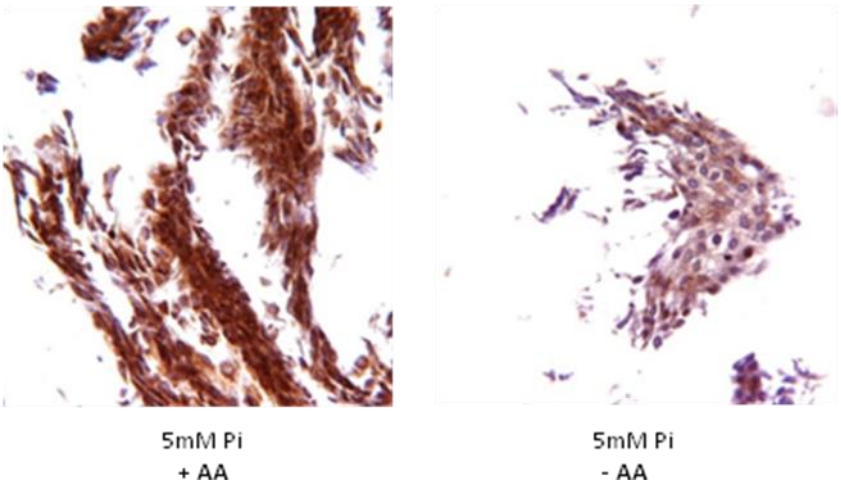

Figure 7: Effect of ascorbic acid on osteonectin expression in high Pi conditions
VSMCs were cultured in calcification medium with 5mM Pi in presence (**A**) or in absence (**B**) of AA for 7 days. Removing AA from the calcification medium results in a significant decrease in osteonectin expression
A: Large amount of cells express diffusely osteonectin (score 4), with strong intensity (score 3), in the cytoplasm (score 2). **B**: Decrease of ostonectin expression with less cells that expressed diffusely osteonectin (score 3), with less strong intensity (score 2), in the cytoplasm (score 2).
Magnification x20. Representative result of one of three different experiments.

6.1.6 Effect of high Pi on the modulation of Osteonectin *ex-vivo*

The observation that osteonectin was expressed in high Pi challenged VSMCs *in vitro* has suggested that it may be involved in the regulation of the osteo/chondrocytic transition of VSMCs *in vivo*, so we decided to study its expression *ex-vivo* in human arteries from a fetus, a 35 years old young adult with hypertension but without macroscopically evident arteries

injury, and from two over 65 years old adults without and with calcified atherosclerotic plaques.

As shown in Figure 8, in human arteries osteonectin was localized, as expected, in VSMCs with a great intensity in fetal life. In the fetal artery, osteonectin has a strong staining (Fig. 8A, and inset). The specimen from the 35 years old patient is an artery with regular architecture, with small signs of fibrosis due to hypertension, considerable as a normal human artery. Osteonectin staining is not very intense compared to the fetal artery (Fig. 8B, and inset). In the atheromatous plaque, both non calcific and calcific, where the muscular layer was displaced by fibrous tissue, osteonectin is present with strong immunoreactivity in residual muscular cells (Fig. 8C-D, and insets).

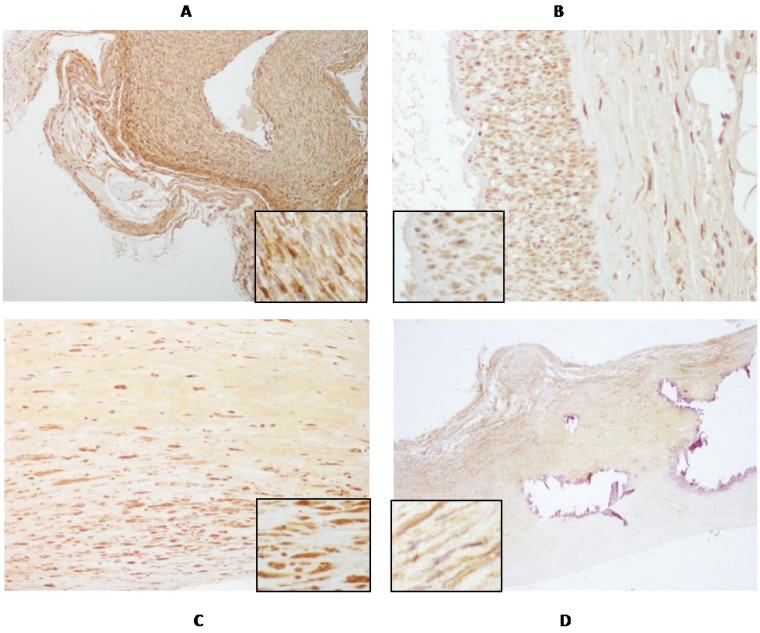

Figure 8: Osteonectin expression in fetal, adult non calcific and adult calcific vessels
A: Artery of a 20th gestational weeks male fetus: strong and diffuse staining in the muscular wall of VSMCs; **B**: Artery from a 35 year old young adult with hypertension but without macroscopically evident artery injury: osteonectin staining is not very intense; **C**: Artery from a 65 year old adult without calcified atheromatous plaques: strong staining in residual VSMCs of aortic wall with atheromatous plaque; **D**: Artery from a 65 year old adult with calcified atheromatous plaques: strong staining in few residual VSMCs of aortic wall with calcified atheromatous plaque. A, B, C: original magnification 20X; D: original magnification 10X; inset: original magnification 40x. Representative result of one of three different experiments.

In order to link osteonectin expression with calcium deposition, we evaluated Von Kossa staining on human arteries. No calcium deposits were present in muscular wall of a 20 weeks fetus (Fig. 9A) and 35 years old patient (Fig. 9B), but we were able to detect calcium

accumulation as fine deposits between the muscle layer of non-calcified atheromatous plaque (Fig. 9C, and inset), and large bulks in calcified plaque (Fig. 9D).

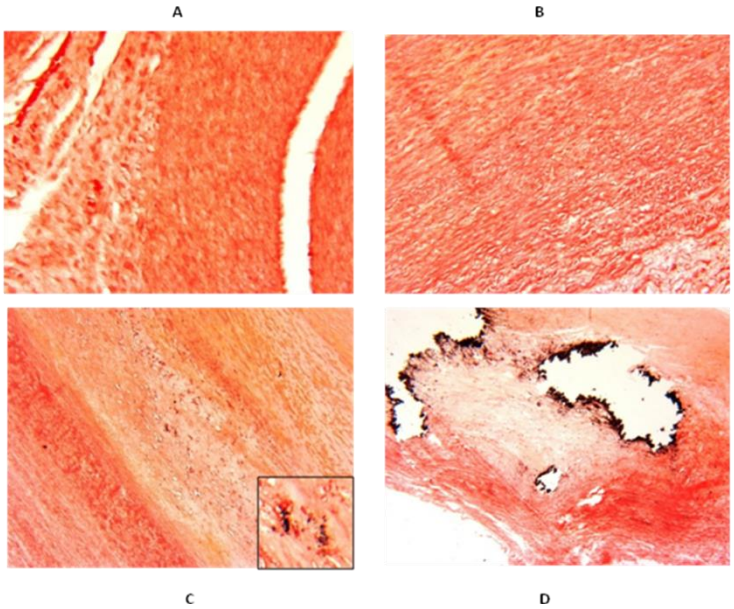

Figure 9: Calcium deposition in embryonic, adult non-calcific and adult calcific vessels
A: Artery of a 20th gestational weeks male fetus: no calcium deposits were present in the muscular wall; **B**: Artery from a 35 year old young adult with hypertension but without macroscopically evident artery injury: no calcium deposits were present in the muscular wall; **C**: Artery from a 65 year old adult without calcified atheromatous plaques: only very few small calcium granules were present in the muscular aortic wall; **D**: Artery from a 65 year old adult with calcified atheromatous plaques: black stained calcium deposits were present in muscular aortic wall with calcified atheromatous plaque. A, B, C, D: original magnification 4X; inset: original magnification 40X. Representative results of one of three experiments.

6.2 PROTECTIVE EFFECTS OF "WASH OUT" TREATMENT ON THE PROGRESSION OF VSMC HIGH Pi-INDUCED CALCIFICATION

6.2.1 "Wash Out" effect on calcium deposition

High Pi concentration (5mM) was able to induce VSMC calcium deposition.

In "Wash Out" experiments, some cells were cultured in continuous presence of high Pi for 7 days, others had a temporary short time suspension of high Pi challenge. Short and repeated high Pi suspension was able to induce an inhibition of calcium deposition in VSMCs, and this protective effect is dependent on the duration of high Pi treatment suspension, with a maximal inhibition when high Pi treatment was suspended for 4 hours ("Pi-4h" condition) every time medium was replaced (2 times in 7 days) (0.55 ± 0.02 ctrl; 0.675 ± 0.62 5mM Pi; 603 ± 0.16 Pi-3h; 478 ± 0.19 Pi-4h; OD/mg protein; *p < 0.01, Fig. 10).

Since 4 hours of high Pi suspension results in a higher inhibition of calcium deposition compared with the 3 hours suspension, in our study we decided to suspend the calcific stimulus for 4 hours every time medium was replaced.

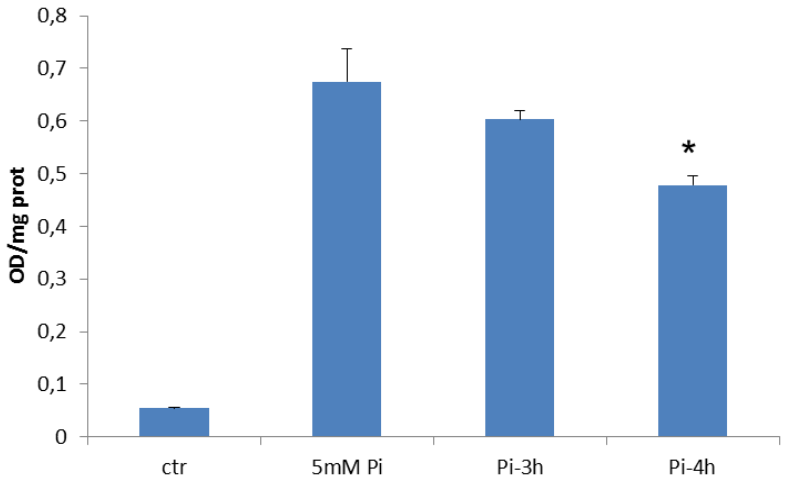

Figure 10: Effect of different time of high Pi suspension on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. The duration of high Pi suspension correlates with the inhibition of calcium deposition. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments. Figure shows a representative experiment (*p < 0.01).

In order to evaluate if the protective effect induced by "Wash Out" treatment was persistent during the progression of calcification, we investigated the effect of this treatment 10 days

after VSMC challenge, with the suspension of high Pi treatment every time medium was replaced (3 times in 10 days).

Our results demonstrate that "Wash Out" significantly inhibits VSMC calcium deposition not only after 7, but, also, after 10 days of high Pi treatment (0.53 ± 0.06 ctrl; 97.42 ± 9.12 5mM Pi; 59.23 ± 5.18 Pi-4h, day 7 of calcification; 0.42 ± 0.03 ctrl; 131.66 ± 18.7 5mM Pi; 68.33 ± 10.53 Pi-4h, day 10 of calcification; µg Ca^{++}/mg protein; *p < 0.01) (Fig. 11).

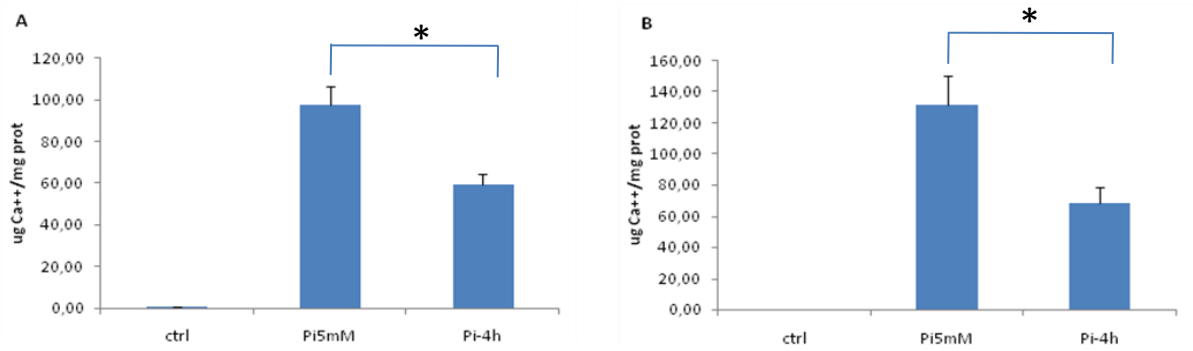

Figure 11: Effect of number of high Pi suspensions on inhibition of calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 (**A**) or 10 (**B**) days, and high Pi treatment was suspended for 4 hours every time medium was replaced. There are no significant differences between the reduction of Ca^{++} deposition in "Wash Out" treatment stopped at day 7 compared to "Wash Out" treatment prolonged for 10 days. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments (*p < 0.01).

Challenging VSMCs with high Pi for 7 days results in calcium deposition, that is detectable by Alizarin Red staining. The positive staining present in high Pi samples was partially inhibited by "Wash Out" treatment (Fig. 12). The semiquantitative analysis confirms a significant decrease in calcium deposition in cells that received a 4 hours high Pi suspension every time medium was replaced (0 ctr, 3.0 ± 0.2 5 mM Pi and 1.2 ± 0.07 Pi-4h; score 0-4) (Fig. 12).

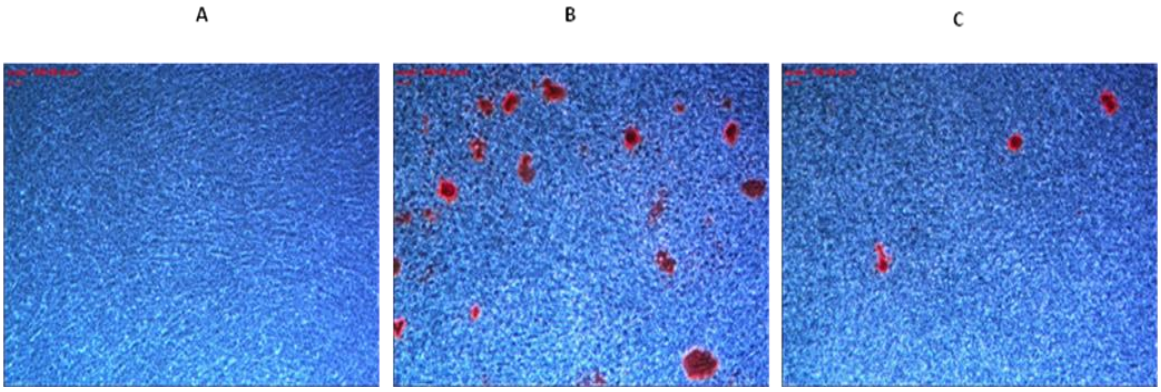

Figure 12: Effect of "Wash Out" on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days and high Pi treatment was suspended for 4 hours every time medium was replaced. Ca^{++} deposition was assessed at light microscopic level by Alizarin Red staining. Control VSMCs (**A**), 5mM Pi (**B**) and Pi-4h (**C**). **A**: No deposit were found in the control culture; **B**: Red color indicates deposits of Ca^{++}-containing mineral; **C:** Decrease in Ca^{++}-containing mineral deposits due to the 4 hours highPi suspension. Magnification x200.
Representative result of one of three different experiments.

To study in detail the effect of "Wash Out" treatment on VSMC calcification, we next analyzed 4 hours high Pi suspension on calcium deposition after 9 days of culture in calcification, evaluating toluidine blue staining on semi-thin slides.

Images show the presence of spreader and larger calcium deposits in VSMCs continuously exposed to high Pi compared to cells that received 4 hours suspension treatment every time medium was replaced, without calcium deposits in control samples, accordingly with alizarin red staining results (Fig. 13).

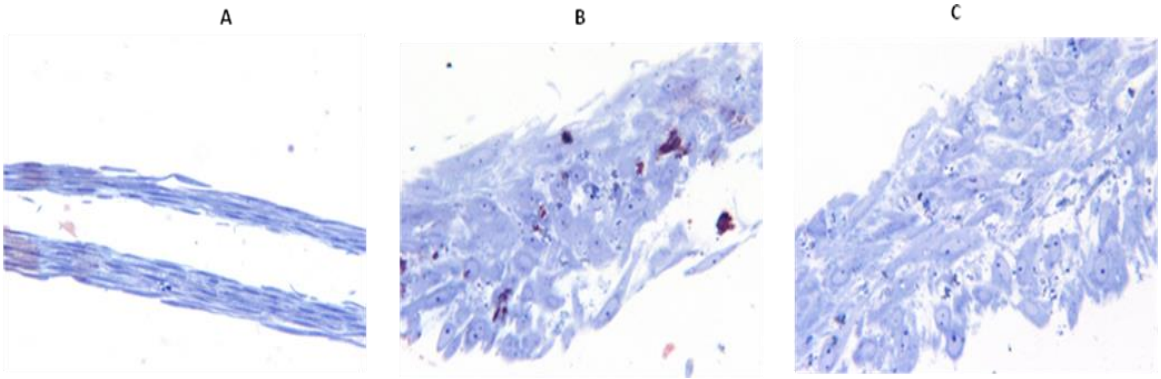

Figure 13: Effect of "Wash Out" on calcium deposition analyzed in semi-thin sections
VSMCs were cultured with 5mM Pi in the calcification medium for 9 days and high Pi treatment was suspended for 4 hours every time medium was replaced. Ca^{++} deposition was assessed level with Toluidine Blue staining. Control VSMCs (**A**), 5mM Pi (**B**) and Pi-4h (**C**). **A**: No deposits were found in the control culture; **B**: Dark blue color indicates Pi-dependent deposits of Ca^{++}-containing mineral; **C:** A decrease in Ca^{++}-containing mineral deposits due to the 4 hours high Pi suspension.
Representative result of one of three different experiments.

6.2.2 Effect of repeated short time PFA treatment on calcium deposition

In order to demonstrate that the protective effect on calcium deposition obtained from 4 hours "Wash Out" treatment is due to the short time absence of high-Pi pro-calcific action on VSMCs, we studied the response of short time phosphonoformic acid (PFA) treatment on calcium deposition in presence of the calcific stimulus 5mM Pi. PFA is a sodium-dependent phosphate co-transporter (Pit-1) and hydroxyapatite formation inhibitor.

During 7 days of high Pi challenge, short and repeated 1mM PFA treatment of VSMCs constantly exposed to high Pi was able to delay the progression of vascular calcification: in VSMCs challenged for 4 hours with 1mM PFA treatment every time medium was replaced there is a significant inhibition of calcium deposition compared to cells continuously exposed to high Pi but not treated with PFA, and this inhibition is comparable with those resulted from "Wash Out" treatment (0.051 ± 0.002 ctrl; 0.787 ± 0.053 5mM Pi; 0.493 ± 0.033 Pi-4h; 0.452 ± 0.038 5mM Pi + 1mM PFA 4h; OD/mg protein; *p < 0.01) (Fig. 14).

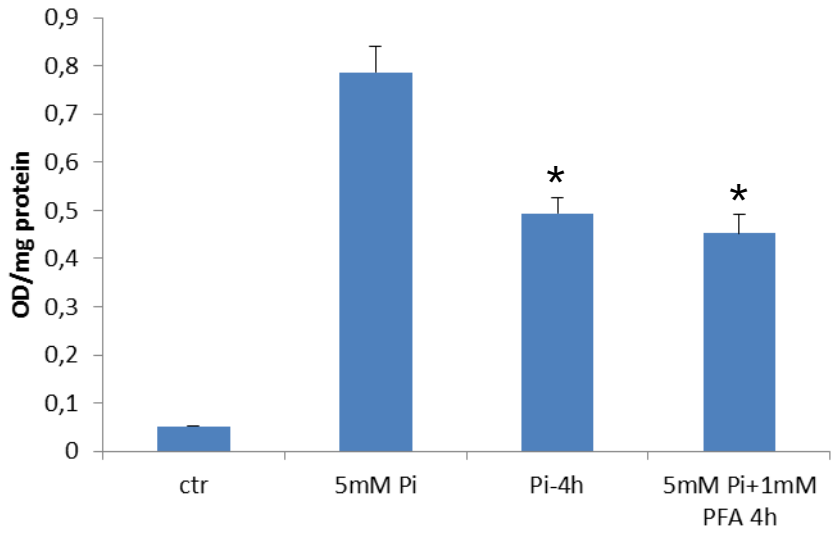

Figure 14: Effect of PFA treatment during "Wash Out" period on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. Some cells were cultured in absence of high Pi for 4 hours every time medium was replaced, and others cells were treated with 1mM PFA for 4 hours every time medium was replaced in continuous presence of high Pi. Ca^{++} deposition was measured and normalized by cellular protein content.
Data were presented as mean ± SE of four different experiments (* p < 0.01).

6.2.3 Effect of repeated short time inhibition of phosphate entry in VSMCs on calcium deposition

In the attempt to understand the relevance of the Pi influx in the "Wash Out" *in vitro* model, we studied calcium deposition after the cells were treated for 4 hours with a saline solution depleted of Na^+ in the presence of the calcific stimulus 5mM Pi ("choline-saline solution").
In VSMCs treated with "choline-saline solution" for 2 hours every time medium was replaced there is a significant inhibition of calcium deposition after 7 days compared to cells constantly cultured in "Na^+-saline solution" in presence of high Pi challenge (0.021 ± 0.001 ctrl; 0.483 ± 0.026 5mM Pi + "Na^+-saline solution"; 0.396 ± 0.021 5mM Pi + "choline-saline solution"; OD/mg protein; * $p < 0.01$) (Fig. 15).

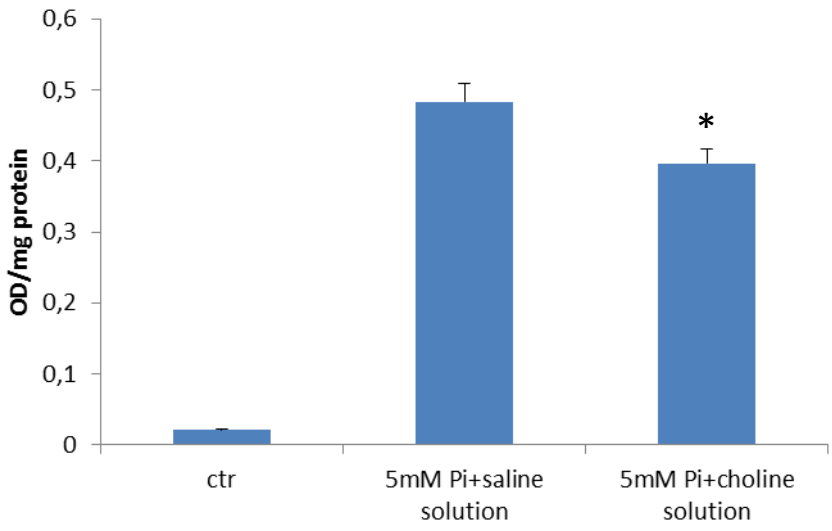

Figure 15: Effects of "choline-saline solution" treatment during "Wash Out" period on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days and calcification medium was replaced with "choline-saline solution" or "Na^+-saline solution" for 2 hours every time medium was replaced. Ca^{++} deposition was measured and normalized by cellular protein content.
Data were presented as mean ± SE of four different experiments (*$p < 0.01$).

Furthermore, to confirm the contribution of Pi influx in VSMCs during the process of vascular calcification, we designed an experimental condition in the attempt to limit PFA action on the blockage of Pi entry in VSMCs through Pit-1. To avoid PFA effect on the formation of hydroxyapatite crystals, we pre-incubated calcification medium with 5mM Pi at 37°C O/N to allow the formation of Ca-P crystals: so, the subsequent addition of PFA can not inhibit the passive deposition of Ca-P hydroxyapatite crystals, because they were pre-formed before

PFA was added. Therefore, in this experimental setting, the effect of PFA on the inhibition of calcium deposition was only the result of inhibition of Pi influx in VSMCs.

In VSMCs constantly exposed to high Pi treated with the "pre-incubated calcification medium" in the presence of 1mM PFA for 4 hours every time medium was replaced there is a partial inhibition of calcium deposition after 7 days compared to cells constantly exposed to high Pi treatment, and this inhibition is comparable to the one resulted from "Wash Out" treatment (0.051 ± 0.002 ctrl; 0.787 ± 0.053 5mM Pi; 0.493 ± 0.033 Pi-4h; 0.497 ± 0.023 5mM Pi "pre-incubated" + 1mM PFA 4h; OD/mg protein; * $p < 0.01$) (Fig. 16).

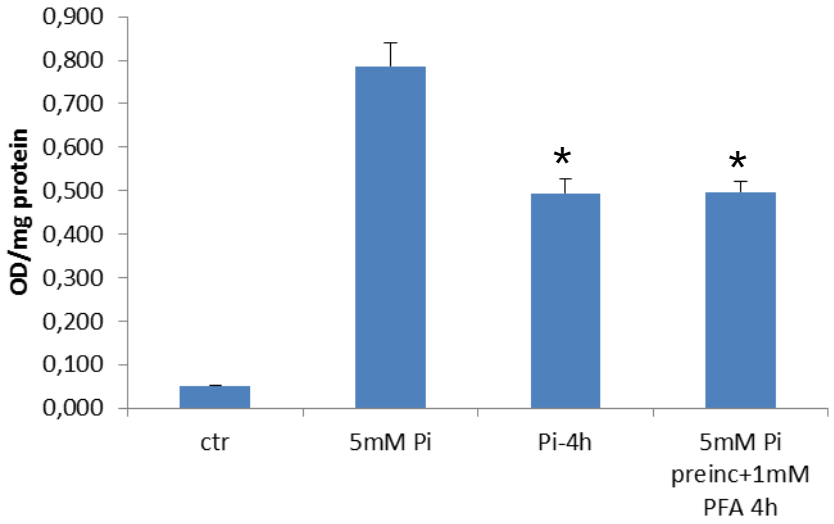

Figure 16: Effect of PFA treatment on the active component of calcium deposition during "Wash Out" period
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. Some cells were cultured in absence of high Pi for 4 hours every time medium was replaced, and other cells were cultured in "pre-incubated calcification medium" with PFA for 4 hours every time medium was replaced. Ca^{++} deposition was measured and normalized by cellular protein content.
Data were presented as mean ± SE of four different experiments (*$p < 0.01$).

6.2.4 Effect of repeated short time free Pi and Ca-P crystals treatments on calcium deposition

To further confirm the contribution of free Pi and to investigate the potential role of Ca-P crystals in VSMCs during the process of calcium deposition, we pre-incubated calcification medium with 5mM Pi at 37°C O/N to allow the formation of Ca-P crystals and we treated VSMCs only with hydroxyapatite crystals or free Pi for 4 hours every time medium was replaced.

In VSMCs constantly exposed to high Pi, but only treated with Ca-P crystals or free Pi for 4 hours every time medium was replaced, there is a partial inhibition of calcium deposition after 7 days compared to cells constantly exposed to high Pi treatment: in particular, both the absence of free Pi and the absence of hydroxyapatite crystals in the "Wash Out" period result in a decrease of calcium deposition that is comparable to "Wash Out" inhibitory effect resulting from the absence of any form of phosphorous for 4 hours every time medium was replaced (0.051 ± 0.002 ctrl; 0.787 ± 0.053 5mM Pi; 0.493 ± 0.033 Pi-4h; 0.491 ± 0.033 Ca-P crystals 4h; 0.532 ± 0.033 free Pi 4h; OD/mg protein; * $p < 0.01$) (Fig. 17).

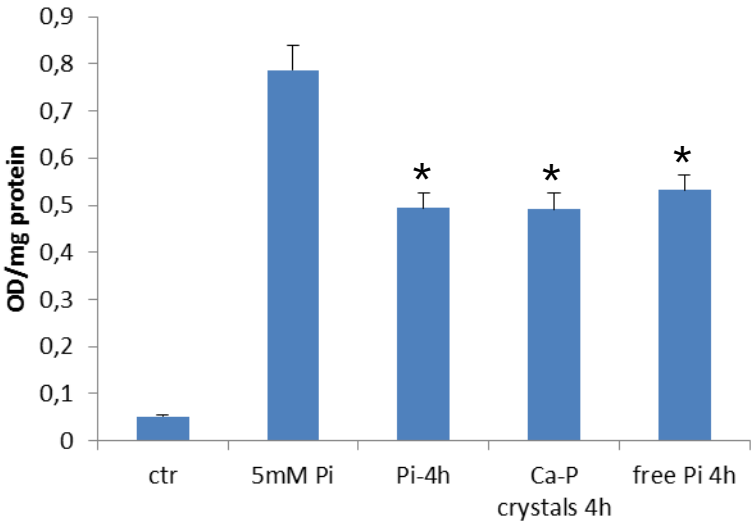

Figure 17: Effect of Ca-P crystals and free Pi during "Wash Out" period on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. Some cells were cultured in absence of high Pi, others only with Ca-P crystals and others only with free Pi for 4 hours every time medium was replaced. Ca^{++} deposition was measured and normalized by cellular protein content.
Data were presented as mean ± SE of four different experiments (*$p < 0.01$).

6.2.5 "Wash Out" effect on the modulation of VSMC lineage markers

In order to elucidate if the prevention in the progression of high Pi-induced calcium deposition by "Wash Out" treatment was associated with an effect on osteoblastic differentiation, we studied the response of 4 hours high-Pi suspension on the expression of VSMC lineage markers, α-actin and SM22α, after challenge with 5mM Pi.

In our model, after 9 days high Pi down-regulates the expression of both α-actin and SM22α, as shown in figure 18 (α-actin 100 ± 5.72% *vs* 62.54 ± 3.40% and SM22α 100 ± 3.11% *vs* 67.36 ± 7.80% of intensity area, control compared with 5mM Pi; *$p < 0.01$) (Fig. 18A-C and

18B-D, respectively). The "Wash Out" treatment preserves the expression of both VSMC lineage markers, compared to the decreased levels of high Pi treated cells (α-actin 62.54 ± 3.40% *vs* 88.08 ± 4.30% and SM22α 67.36 ± 7.8% *vs* 90.03 ± 5.59% of intensity area, 5mM Pi compared with Pi-4h; *p < 0.01) (Fig. 18A-C and 18B-D, respectively).

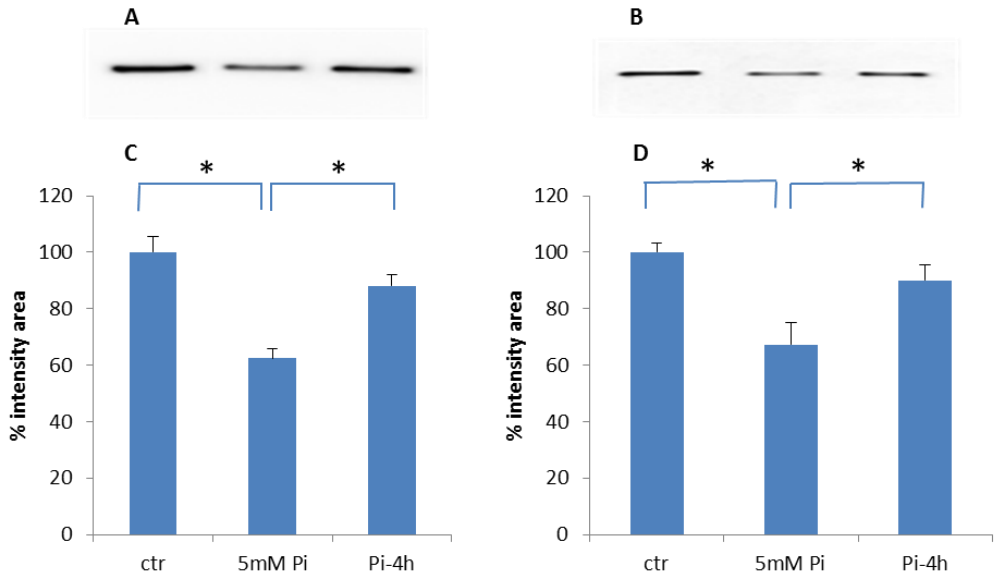

Figure 18: Effects of "Wash Out" on VSMC markers expression
VSMCs were cultured with 5mM Pi in calcification medium for 9 days, and high Pi treatment was suspended for 4 hours every time medium was replaced. α-actin (panel **A**) and SM22α (panel **B**) protein expression was detected by immunoblotting analysis. **Lane 1**: control VSMCs; **lane 2**: 5mM Pi; **lane 3**: Pi-4h. Analysis of the intensity of the bands was shown for α-actin (panel **C**) and SM22α (panel **D**). "Wash Out" prevents VSMC markers down-regulation induced by high Pi. Representative bands of one of four different experiments. Data were presented as mean ± SE of four different experiments (*p < 0.01).

6.2.6 "Wash Out" effect on VSMC apoptosis

Since several studies demonstrated that apoptosis is an important promoter of VSMC calcification, we tried to understand if "Wash Out" treatment could affect vascular mineralization as a consequence of a decrease of cell death.

6.2.6.1 "Wash Out" effect on VSMC DNA fragmentation

First of all, we analyzed DNA fragmentation after challenge of VSMCs with 5mM Pi for 7 days or with 4 hours suspension of high Pi treatment every time medium was replaced. During calcification process, high Pi increases cytoplasmic histone-associated DNA fragments, and these increase was partially inhibited after the short time high Pi suspension (1.00 ± 0.17 ctrl; 2.92 ± 0.31 5mM Pi; 2.19 ± 0.08 Pi-4h; enrichment factor of apoptotic fragments; *p <

0.01) (Fig. 19). Then, we investigated if the "Wash Out" protection could be preserved at a later time point of the calcification process. In particular, at day 11 we obtained an increased inhibition of DNA fragmentation compared with day 7 (1.00 ± 0.17 ctrl; 3.18 ± 0.09 5mM Pi; 1.92 ± 0.11 Pi-4h; enrichment factor of apoptotic fragments; *p < 0.01) (Fig. 19).

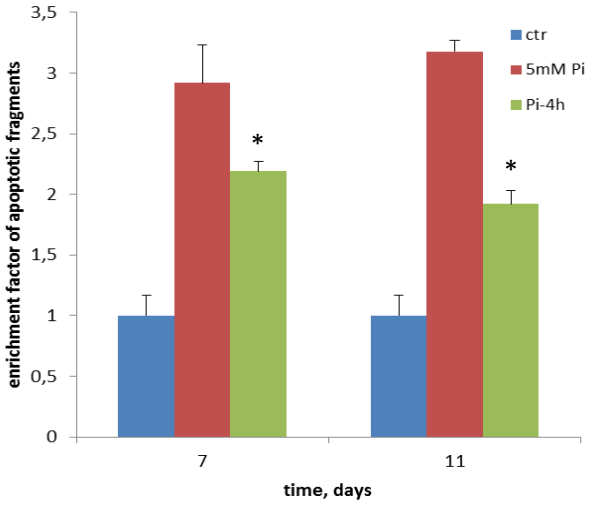

Figure 19: Effects of "Wash Out" on VSMC DNA fragmentation
VSMCs were cultured with 5mM Pi in calcification medium for 7 or 11 days, and high Pi treatment was suspended for 4 hours every time medium was replaced. "Wash Out" prevents VSMC DNA fragmentation during the progression of vascular calcification.
Data were presented as mean ± SE of four different experiments (*p < 0.01).

6.2.6.2 "Wash Out" effect on the modulation of VSMC anti-apoptotic protein Axl expression

Since apoptosis is the result of the activation of different molecular pathways, we decided to investigate if "Wash Out" treatment could involve the modulation of the anti-apoptotic and pro-survival marker Axl. In our model, challenge with 5mM Pi for 9 days results in a decreased expression of Axl, and the suspension of 5mM Pi for 4 hours every time medium was replaced fails to restore Axl expression at levels similar to those observed in control cells (69.41 ± 1.81% *vs* 72.63 ± 1.26% of intensity area, 5mM Pi compared with Pi-4h; n.s.) (Fig. 20).

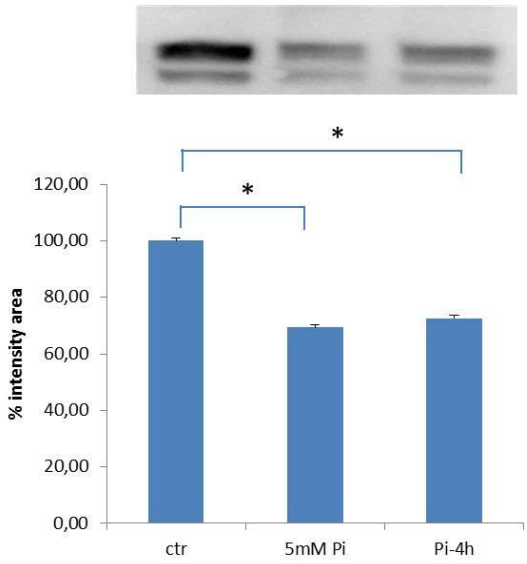

Figure 20: Effects of "Wash Out" on VSMC Axl expression
VSMCs were cultured with 5mM Pi in calcification medium for 9 days, and Pi treatment was suspended for 4 hours every time medium was replaced. "Wash Out" does not prevent the high Pi-induced down regulation of Axl expression. **Lane 1**: control VSMCs; **lane 2**: 5mM Pi; **lane 3**: Pi-4h.
Data are presented as a representative experiment out of four (*p < 0.01).

6.2.7 "Wash Out" effect on VSMC autophagy

Since autophagy seems to be a mechanism involved in vascular calcification, we decided to investigate its potential modulation in "Wash Out" treatment. Autophagy is a common mechanism adopted by all cells for self-digestion and to degrade cellular organelles.

6.2.7.1 Electron microscopy analysis

Electronic microscopy images show that in cells continuously challenged with high Pi there are both calcific and healthy mithocondria: calcific mithocondria are more electron-dense, completely calcified and loss their structural morphology (Fig. 21A). Cells increment the expulsion of these calcific mithocondria (Fig. 21B) and the deposition of hydroxyapatite crystals at extracellular level (Fig. 21C).

In cells that received the 4 hours high Pi suspension every time medium was replaced there is an increase of the autophagy: damaged mitochocondria are isolated by the cell into intracytoplasmatic double-membrane vesicles, that, probably, will be targeted to lysosome for digestion (Fig. 21D, E).

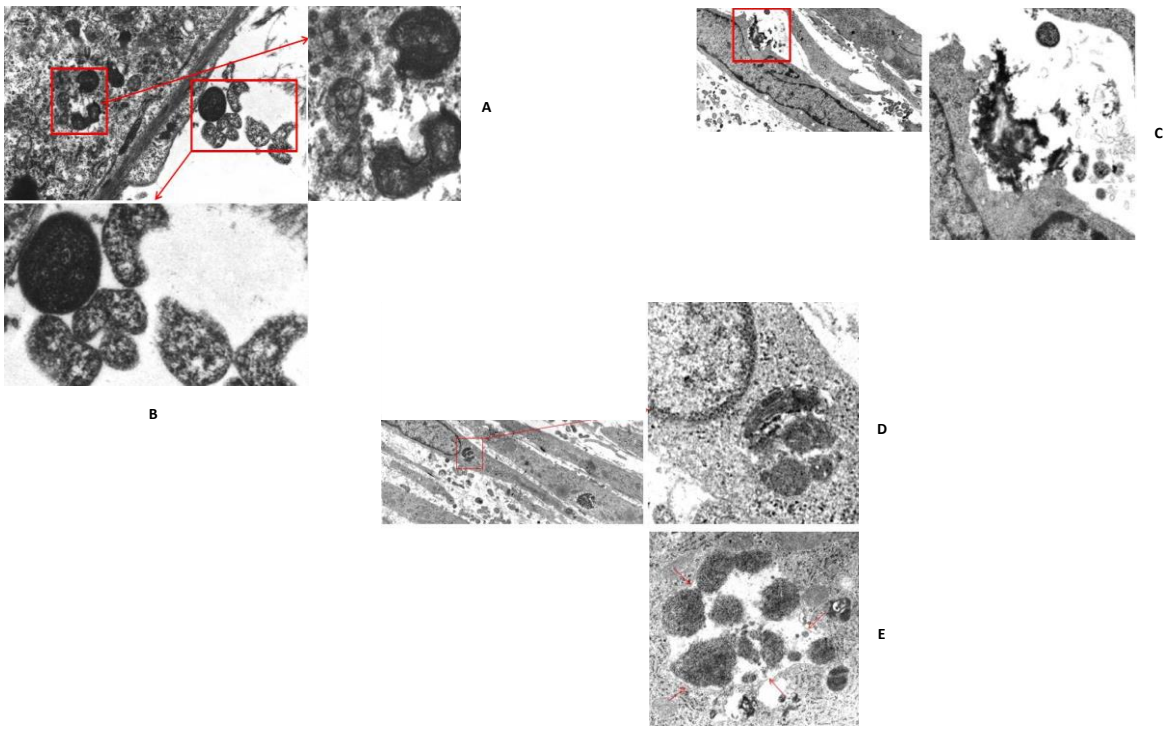

Figure 21: Effects of "Wash Out" on VSMC autophagy
VSMCs were cultured with 5mM Pi in calcification medium for 9 days, and Pi treatment was suspended for 4 hours every time medium was replaced. Arrows indicate (**A**) a representative calcific mithocondria, (**B**) extracellular vescicles rich in calcific mitochondria and (**C**) an extracellular hydroxyapatite deposit in cells continuously challenged with high Pi. Panels **D** and **E** represent autophagic intracellular vacuoles rich in damaged mitochondria in cells that received "Wash Out" treatment.
A, B: 12,000x original magnification (insets from a 8,000x original magnification); C: 8,000x original magnification (inset from a 2,500x original magnification); D, E: 10,000x original magnification (insets from a 4,000x original magnification).

6.2.7.2 "Wash Out" effect on the modulation of VSMC autophagocitic protein LC3-II expression

To confirm electron microscopy results, we decided to investigate if "Wash Out" treatment could involve the modulation of LC3-II autophagic marker.

At the beginning, we analyzed LC3-II expression at day 9, and we found higher levels in high phosphate-challenged cells compared to control and "Wash Out"-treated cells (Fig. 22A). Then, since autophagy is a cell dynamic process, we decided to treat cells with chloroquine, an agent that impairs lysosomal acidification with the consequent blockage of LC3-II turnover, in order to freeze the autophagic flux at day 9. After the treatment of cells with this agent, we found that control and "Wash Out"-treated VSMCs show an increased expression of LC3-II compared to the condition "absence of chloroquine", whereas cells

challenged with 5mM Pi for 9 days do not modulate LC3-II expression in presence of chloroquine (Fig. 22B).

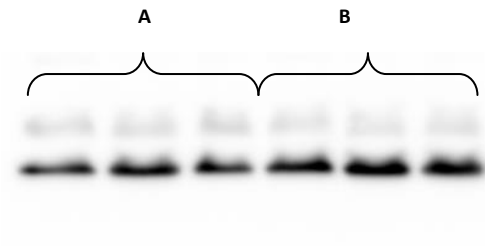

Figure 22: Effects of "Wash Out" on VSMC LC3-II expression
VSMCs were cultured with 5mM Pi in calcification medium for 9 days, and Pi treatment was suspended for 4 hours every time medium was replaced. **A**: Cells not treated with chloroquine: high Pi-treated cells express higher levels of LC3-II compared to control and "Wash Out"-treated cells **B**: cells treated with chloroquine: control and "Wash Out"-treated up-regulate LC3-II expression.
Lane 1: control VSMCs; **lane 2**: 5mM Pi; **lane 3**: Pi-4h; **Lane 4**: control VSMCs+25µM chloroquine; **lane 5**: 5mM Pi+25µM chloroquine; **lane 6**: Pi-4h+25µM chloroquine.
Data are presented as a representative preliminary experiment.

6.3 EFFECT AND MECHANISMS OF ACTION OF LANTHANUM CHLORIDE ($LaCl_3$), GADOLINIUM CHLORIDE ($GdCl_3$) AND CALINDOL ON THE DELAY OF THE PROGRESSION OF HIGH Pi-INDUCED VASCULAR CALCIFICATION

6.3.1 Effect of $LaCl_3$, $GdCl_3$ and Calindol on high Pi-induced VSMC calcium deposition

VSMCs calcify after challenge with high Pi concentration (5mM). Calcium deposition is concentration-dependently inhibited by addition of $LaCl_3$, with a maximal effect at 100µM at day 7 of calcification (0.5 ± 0.12 ctr, 46.51 ± 2.51 5mM Pi, 32.82 ± 5.28 2µM $LaCl_3$, 30.1 ± 2.55 10µM $LaCl_3$, 20.12 ± 1.33 50µM $LaCl_3$, 19.92 ± 1.28 100µM $LaCl_3$; µg Ca^{++}/mg protein; *p < 0.01) (Fig. 23).

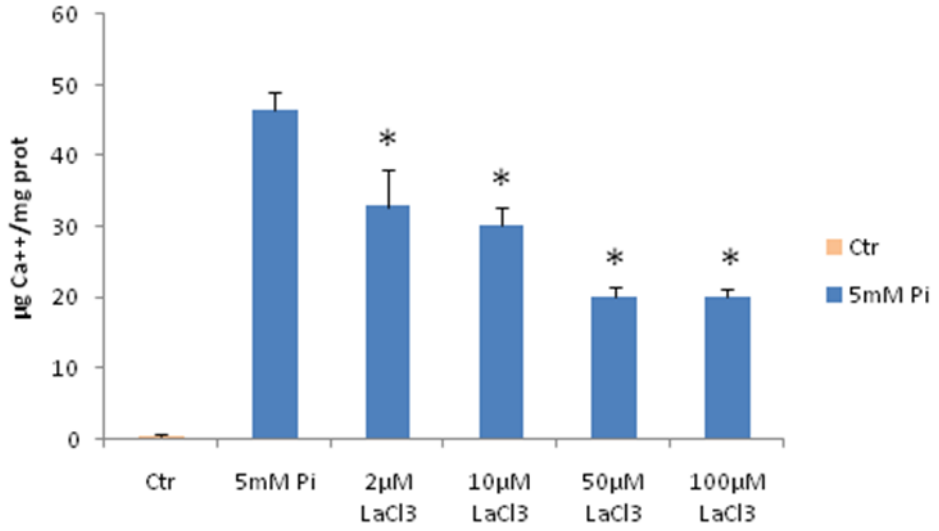

Figure 23: Effect of lanthanum on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. The presence of increasing concentration of lanthanum in the calcification medium induces a decrease in high Pi-induced Ca^{++} levels, that is statistically significant compared to high Pi alone. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments (*p < 0.01).

Moreover, $GdCl_3$ inhibits calcium deposition, with a maximal effect at 50µM at day 7 of calcification (0.5 ± 0.11 ctr, 34.02 ± 2.53 5mM Pi, 30.5 ± 1.34 2µM $GdCl_3$, 24.6 ± 2.52 10µM $GdCl_3$, 16.97 ± 2.12 50µM $GdCl_3$; µg Ca^{++}/mg protein; *p < 0.01) (Fig. 24), that is comparable with the inhibitory effect of $LaCl_3$ compared to cells challenged with high Pi in absence of any pharmacological treatment. It was not possible to increase $GdCl_3$ concentration above 50µM due to induction of cell toxicity.

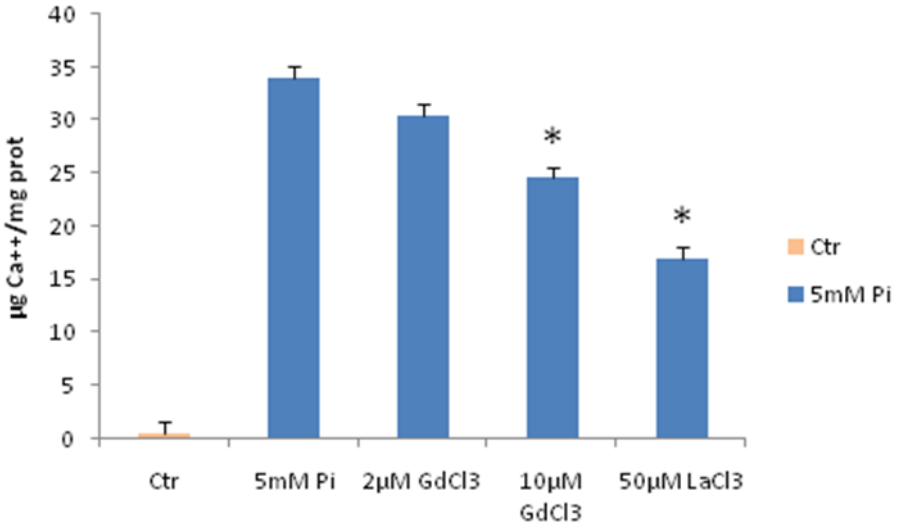

Figure 24: Effect of gadolinium on calcium deposition

VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. The presence of increasing concentration of gadolinium in the calcification medium induces a decrease in high Pi-induced Ca^{++} levels, that is statistically significant compared to high Pi alone. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments (*p < 0.01).

The calcimimetic calindol inhibits calcium deposition concentration-dependently, with a maximal inhibition achieved at 100nM the highest concentration tested, since it was not possible enhance the concentration for cell toxicity induction (0.5 ± 0.11 ctr, 53.6 ± 5.32 5mM Pi, 46.66 ± 6.09 10nM calindol, 37.23 ± 3.26 50nM calindol, 13.52 ± 2.04 100nM calindol; µg Ca^{++}/mg protein; *p < 0.01) (Fig. 25).

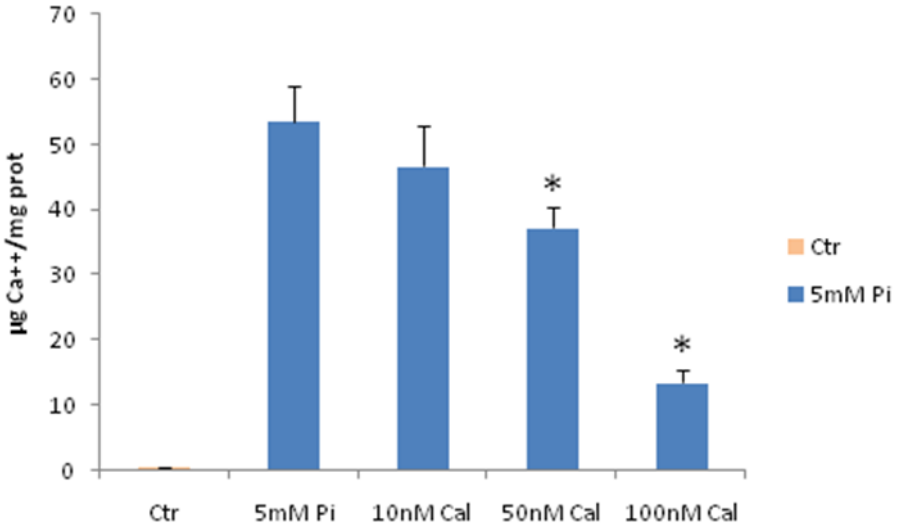

Figure25: Effect of calindol on calcium deposition

VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. The presence of increasing concentration of calindol in the calcification medium induces a decrease in high Pi-induced Ca^{++} levels, that is statistically significant compared to high Pi alone. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments (*p < 0.01).

Challenging VSMCs with high-Pi for 7 days results in calcium deposition, detectable by Alizarin Red staining. The positive staining present in high Pi samples was inhibited by the addition of 100µM $LaCl_3$, 50µM $GdCl_3$ and 50nM calindol, and the semiquantitative analysis confirms that the decrease in calcium deposits is significant (0 ctr, 3.2 ± 0.1 5mM Pi, 1.8 ± 0.1 100µM $LaCl_3$, 1.9 ± 0.1 50µM $GdCl_3$, 2.0 ± 0.2 50nM calindol, respectively; score 0-4; *p < 0.01) (Fig. 26).

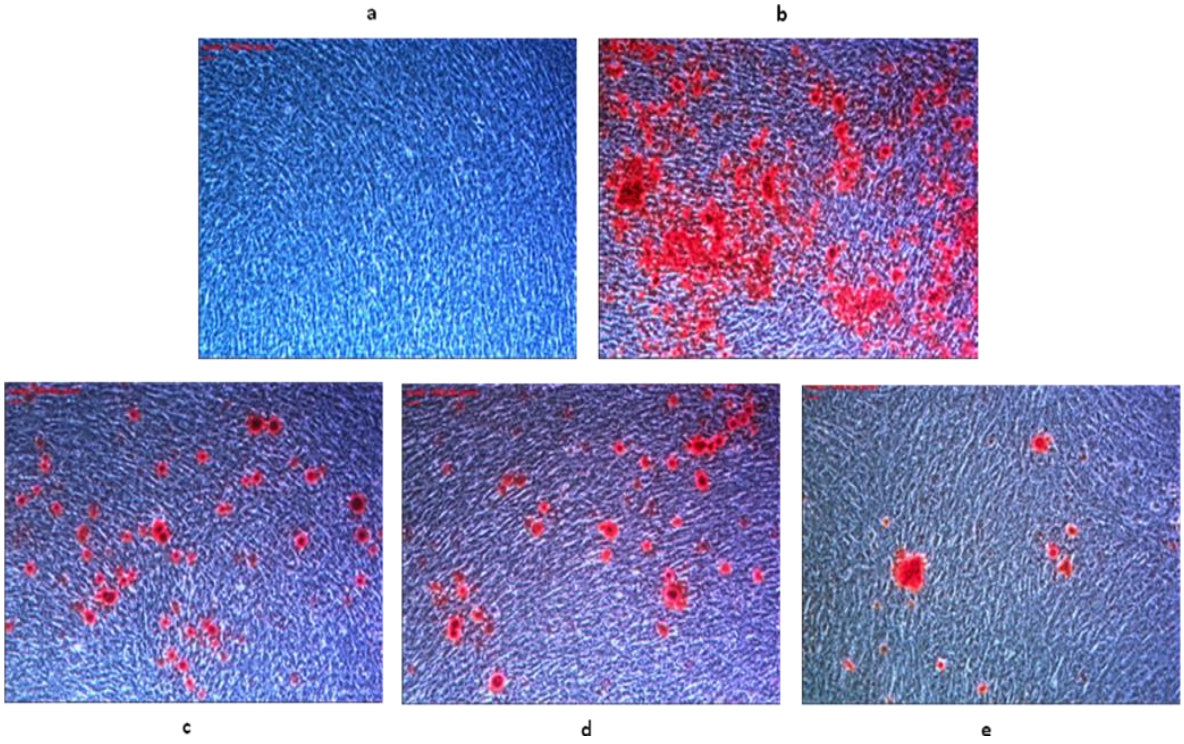

Figure 26: Effect of lanthanum, gadolinium and calindol on calcium deposition
VSMCs were cultured with 5mM Pi in the calcification medium for 7 days. Ca^{++} deposition was assessed at light microscopic level by Alizarin Red staining. Control VSMCs (**a**), 5mM Pi (**b**), 100µM $LaCl_3$ (**c**) 50µM $GdCl_3$ (**d**) and 50nM calindol (**e**). **a**: No deposit were found in the control culture; **b**: Red color indicates high Pi-dependent deposits of Ca^{++}-containing mineral; **c**, **d** and **e** show a decrease in Ca^{++}-containing mineral deposits due to the inhibition of high Pi-induced calcium deposition by lanthanum, gadolinium and calindol.
Magnification x200. Representative result of one of three different experiments.

In order to evaluate if the prevention in the progression of high Pi-induced calcium deposition promoted by $LaCl_3$ and $GdCl_3$ was persistent at a later time point of calcification, we investigated the potential effect of these compounds 15 days after challenge with 5mM

Pi, finding that both 100µM $LaCl_3$ and 50µM $GdCl_3$ significantly inhibit calcium deposition (3 ± 0.1 ctr, 115.43 ± 5.85 5mM Pi, 63.1 ± 5.02 100µM $LaCl_3$, 44.68 ± 4.57 50µM $GdCl_3$; µg Ca^{++}/mg protein *p < 0.01) (Fig. 27A).

After challenge with high Pi for 15 days, VSMCs show a positive staining with Alizarin Red compared to controls (Fig. 27, B, C), which results inhibited by the addition of 100µM $LaCl_3$ and 50µM $GdCl_3$ (Fig. 27, D, E). A semiquantitative analysis confirms a significant decrease in calcium deposition in presence of $LaCl_3$ and $GdCl_3$ (3.8 ± 0.1 5mM Pi, 2.1 ± 0.1 100µM $LaCl_3$ and 2.0 ± 0.1 50µM $GdCl_3$; score 0-4; *p < 0.01).

The protective role of lanthanum and gadolinium on calcium deposition is independent by any chelating effect on phosphate, since Pi levels in the culture medium result unchanged after $LaCl_3$ and $GdCl_3$ addition at the highest concentration (*data not shown*).

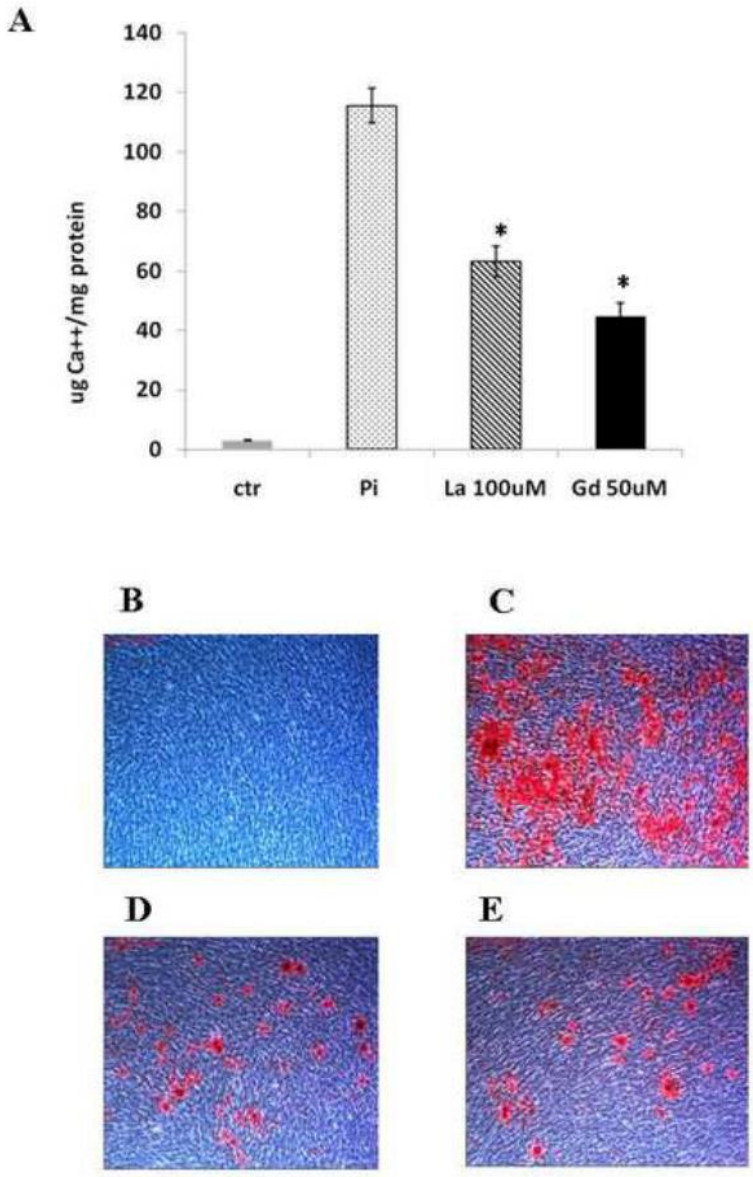

Figure 27: Effects of lanthanum and gadolinium on calcium deposition in the progression of vascular calcification

VSMCs were cultured with 5mM Pi in the calcification medium for 15 days with 100µM lanthanum or 50µM gadolinium. **A**: Both lanthanum and gadolinium induce a decrease in high Pi-induced Ca^{++} levels that is statistically significant compared to high Pi alone. Ca^{++} deposition was measured and normalized by cellular protein content. Data were presented as mean ± SE of four different experiments (*$p < 0.01$).
B-E: Control VSMCs (**B**), 5mM Pi (**C**), 100µM lanthanum (**D**) and 50µM gadolinium (**E**). Ca^{++} deposition was assessed at light microscopic level by Alizarin Red staining. **B**: No deposits were found in the control culture; **C**: Red color indicates high Pi-dependent deposits of Ca^{++}-containing mineral; **D** and **E** show a decrease in Ca^{++}-containing mineral deposits due to the inhibition of high Pi-induced calcium deposition by lanthanum and gadolinium. Magnification x200. Representative result of one of three different experiments.

6.3.2 Effect of $LaCl_3$, $GdCl_3$ and Calindol on high Pi-induced VSMC osteoblastic differentiation

6.3.2.1 Modulation of VSMC lineage markers

In order to elucidate if the prevention in the progression of high Pi-induced calcium deposition by $LaCl_3$, $GdCl_3$ and calindol was associated with an effect on osteoblastic differentiation, we studied the effect of these three compounds on the expression of VSMC markers, α-actin and SM22α, after challenge with 5mM Pi.

In our model, after 8 days high Pi is able to down-regulate the expression of both α-actin (100 ± 1% vs. 76.5 ± 1.4% of intensity area, control compared with 5mM Pi; *p < 0.01) (Fig. 28) and SM22α (100.0 ± 0.3% vs. 85.7 ± 2.9% of intensity area, control compared with 5mM Pi; *p < 0.01) (Fig. 29).

$LaCl_3$, $GdCl_3$ and calindol show different effects on the modulation of α-actin and SM22α. In fact, the administration of $LaCl_3$ preserves the expression of both VSMC lineage markers, compared to the decreased levels of high Pi treated cells (α-actin 76.5 ± 1.4% *vs* 103.9 ± 2.0% (Fig. 28A) and SM22α 85.7 ± 2.9% *vs* 95.0 ± 4.6% of intensity area (Fig. 29A), 5mM Pi compared with 100µM $LaCl_3$; *p < 0.01). In contrast, $GdCl_3$ and calindol don't affect high Pi-induced VSMC markers down-regulation (α-actin 76.5 ± 1.4% *vs* 60.9 ± 2.0% (Fig. 28A) and SM22α 85.7 ± 2.9% vs. 68.9 ± 6.2% of intensity area (Fig. 29A), 5mM Pi compared with 50µM $GdCl_3$; α-actin 76.5 ± 1.4% *vs* 72.0 ± 2.7% (Fig. 28B) and SM22α 85.7 ± 2.9% *vs* 71.7 ± 1.6% of intensity area (Fig. 29B), 5mM Pi compared with 50nM calindol; n.s.).

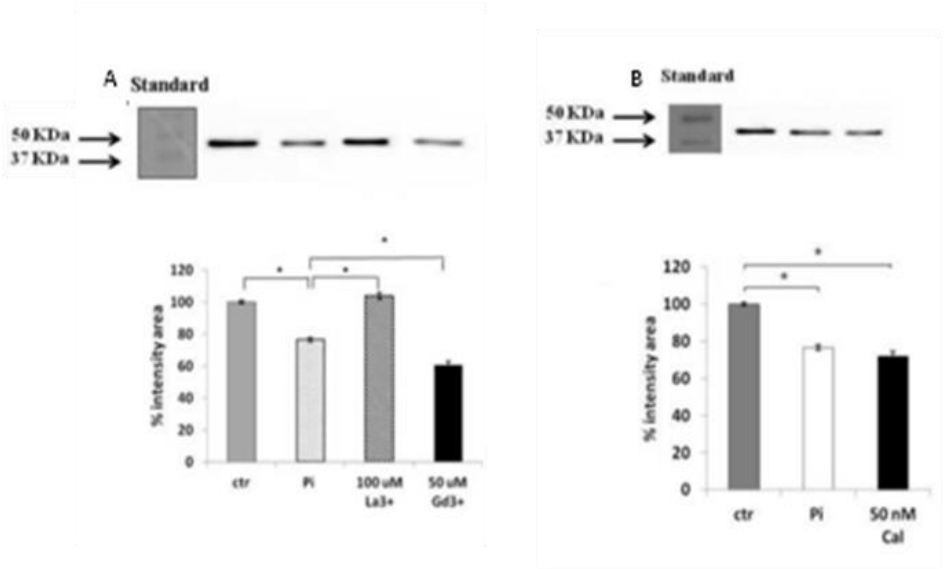

Figure 28: Effects of lanthanum, gadolinium and calindol on VSMC α-actin expression

VSMCs were cultured for 8 days in calcification medium with 100µM lanthanum, 50µM gadolinium or 50nM calindol and challenged with 5mM Pi. Protein expression was detected by immunoblotting analysis. **A. Lane 1**: Control VSMCs; **lane 2**: 5mM Pi; **lane 3**: 100µM $LaCl_3$; **lane 4**: 50µM $GdCl_3$. **B. Lane 1**: Control VSMCs; **lane 2**: 5mM Pi; **lane 3**: 50nM calindol. Analysis of the intensity of the bands is shown for α-actin.
Lanthanum prevents, whereas gadolinium and calindol do not affect VSMC α-actin down-regulation induced by high Pi. Representative bands of one of four different experiments.
Data were presented as mean ± SE of four different experiments (*p < 0.01).

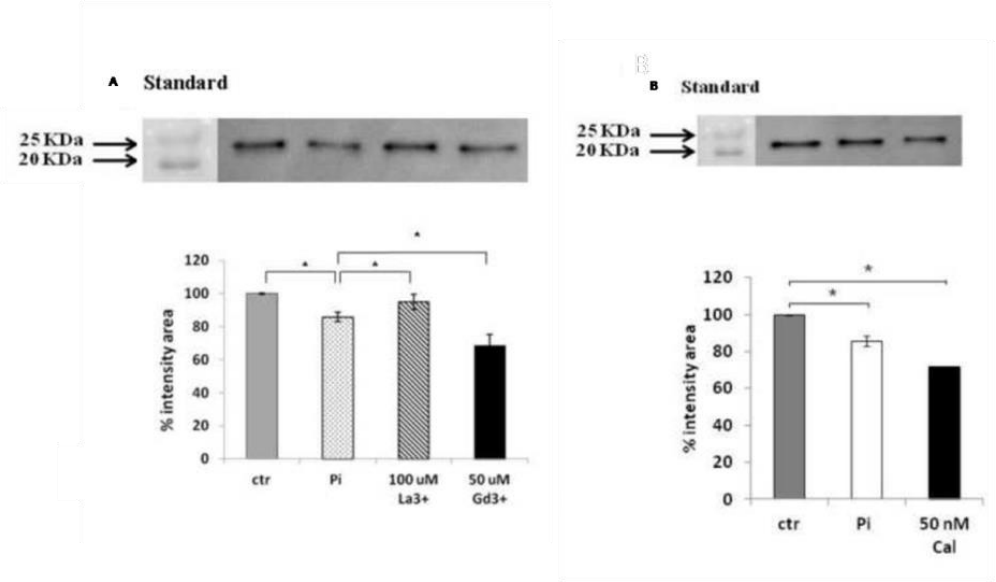

Figure 29: Effects of lanthanum, gadolinium and calindol on VSMC SM22α expression
VSMCs were cultured for 8 days in calcification medium with 100µM lanthanum, 50µM gadolinium or 50nM calindol and challenged with 5mM Pi. Protein expression was detected by immunoblotting analysis. **A. Lane 1**: Control VSMCs; **lane 2**: 5mM Pi; **lane 3**: 100µM $LaCl_3$; **lane 4**: 50µM $GdCl_3$. **B. Lane 1**: Control VSMCs; **lane 2**: 5mM Pi; **lane 3**: 50nM calindol. Analysis of the intensity of the bands is shown for SM22α.
Lanthanum prevents, whereas gadolinium and calindol do not affect VSMC SM22α down-regulation induced by high Pi. Representative bands of one of four different experiments. Data were presented as mean ± SE of four different experiments (*p < 0.01).

To study how the modulation of VSMC markers was affected during the progression of calcification, we tested the expression of α-actin and SM22α at later time points.

As shown in figure 30, also at day 9 and 12, after high Pi challenge, $LaCl_3$ preserves both α-actin and SM22α expression, compared to high Pi-treated cells (α-actin 75.4 ± 2.7% *vs* 113.5 ± 2.8% and 103.3 ± 2.3%, SM22α 80.9 ± 0.4% *vs* 93.3 ± 0.6% and 95.2 ± 0.4% of intensity area, 5mM Pi compared to 100µM $LaCl_3$ at day 9 and at day 12, respectively; *p < 0.01). In contrast, with the progression of vascular calcification, $GdCl_3$ fails to restore the down-regulation of both VSMC markers. The expression of both VSMC markers after challenge with 5mM Pi is not different between day 8 and day 12 (*data not shown)*.

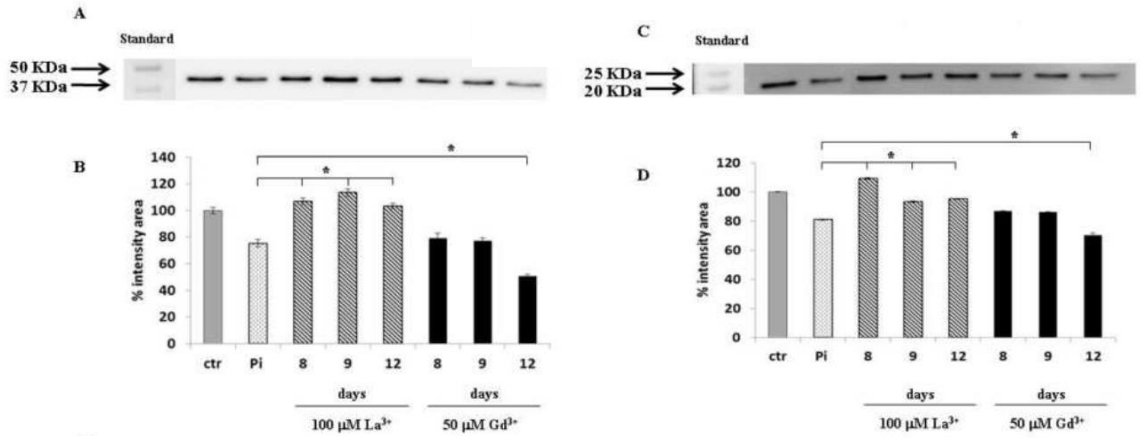

Figure 30: Effects of lanthanum and gadolinium on VSMC markers expression during the progression of vascular calcification

VSMCs were cultured for 8, 9 or 12 days in calcification medium with 100µM lanthanum or 50µM gadolinium and challenged with 5mM Pi. α-actin (**A**) and SM22α (**C**) protein expression was detected by immunoblotting analysis. **Lane 1**: Control VSMCs (8 days); **lane 2**: 5mM Pi (8 days); **lane 3-4-5**: 100µM $LaCl_3$ 8, 9 and 12 days respectively; **lane 6-7-8**: 50µM $GdCl_3$ 8, 9 and 12 days, respectively.

During progression of calcification lanthanum prevents, whereas gadolinium fails to restore the VSMC markers down-regulation induced by high Pi. Analysis of the intensity of the bands is shown for α-actin (**B**) and SM22α (**D**). Representative bands of one of three different experiments.

Data were presented as mean ± SE of three different experiments (*p < 0.01).

6.3.2.2 Modulation of VSMC osteoblastic genes and proteins expression

We next analyzed the effect of $LaCl_3$, $GdCl_3$ and calindol on BMP-2, Cbfα1/RUNX-2, MGP gene expression, all markers involved in the osteoblastic differentiation process of VSMCs, and the influence of $LaCl_3$ and $GdCl_3$ on osteonectin protein expression (SPARC).

The time course experiment shows that, after 2 hours of incubation, the challenge with 5mM Pi results in a peak with a 5.35 ± 0.98 fold increase in BMP-2 mRNA expression compared to the relative expression in normal VSMCs (t_0 1.01 ± 0.06) (*p < 0.01) (Fig. 31A); no increase was observed at later time points (from 1 to 7 days of calcification, *data not shown*).

Figure 31B shows that the pretreatment with $LaCl_3$, $GdCl_3$ and calindol partially inhibits the increase in BMP-2 mRNA expression induced by high Pi (4.66 ± 0.49 5mM Pi, 3.49 ± 0.14 100µM $LaCl_3$, 3.41 ± 0.36 50µM $GdCl_3$ and 2.94 ± 0.43 50nM calindol relative expression; *p < 0.01).

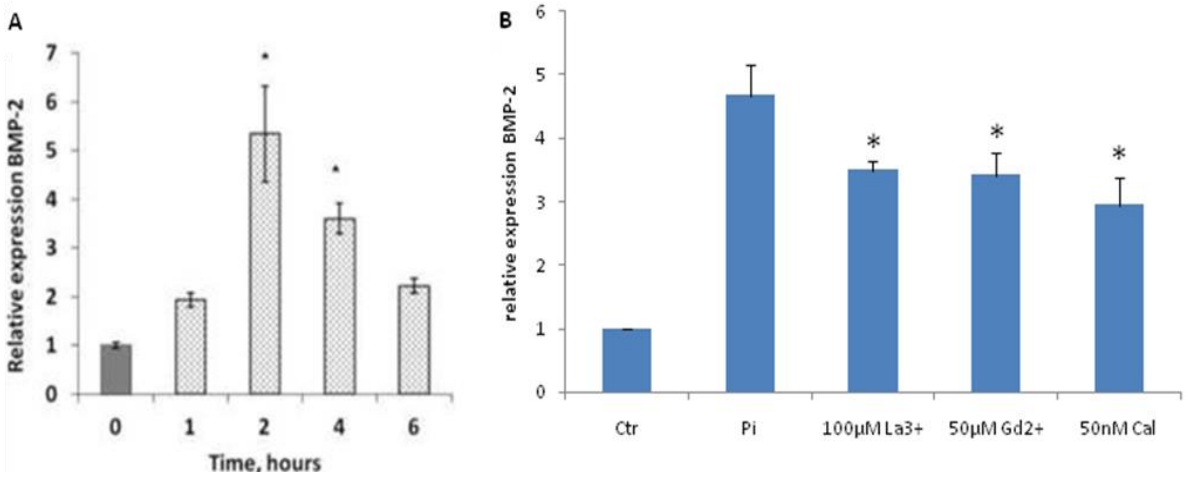

Figure 31: Effects of lanthanum, gadolinium and calindol on BMP-2 gene expression
VSMCs were cultured in calcification medium and BMP-2 mRNA expression was measured by RT-PCR. **A**: Time course of BMP-2 expression after challenge with 5mM Pi. After two and four hours of high Pi challenge there is a significant increase in BMP-2 mRNA levels compared to the relative expression in normal VSMCs (t_0). **B**: Effect of lanthanum, gadolinium and calindol on BMP-2 gene expression. Pretreatment with 100µM $LaCl_3$, 50µM $GdCl_3$ and 50nM calindol induces a significant decrease in BMP-2 mRNA levels two hours after high Pi challenge. Data were presented as mean ± SE of three different experiments (*p < 0.01).

Cbfα1/RUNX-2 mRNA expression has a peak 4 hours after high Pi challenge (1.65 ± 0.06 5mM Pi, relative expression; *p < 0.01, Fig. 32A). Interestingly, no changes in Cbfα1/RUNX-2 mRNA levels were detectable after $LaCl_3$, $GdCl_3$ and calindol pretreatment compared with the increase after high Pi challenge (Fig. 32B).

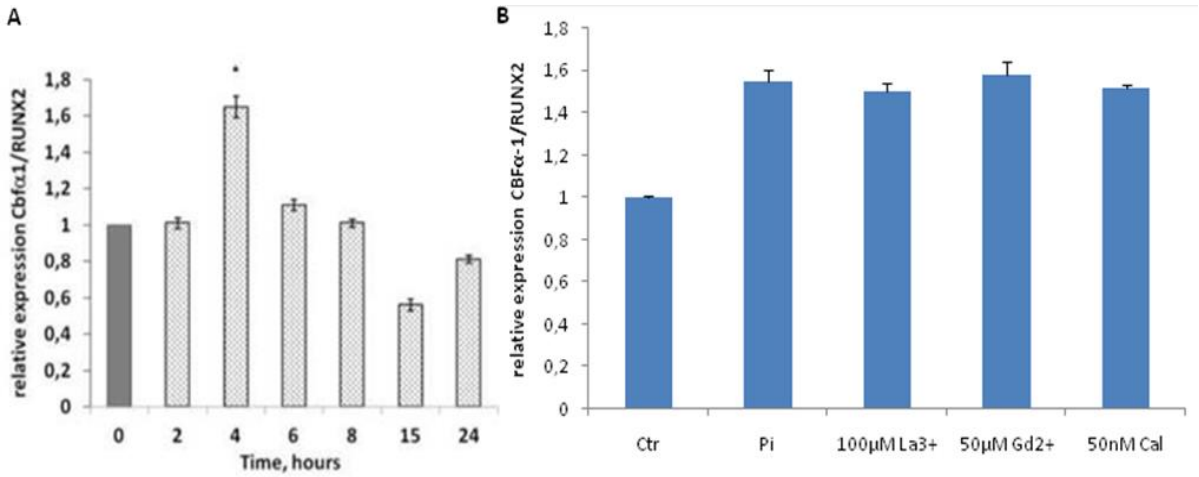

Figure 32: Effects of lanthanum, gadolinium and calindol on Cbfα1/RUNX2 gene expression
VSMCs were cultured in calcification medium and Cbfα1/RUNX2 mRNA expression was measured by RT-PCR. **A**: Time course of Cbfα1/RUNX2 expression after challenge with 5mM Pi. Four hours after high Pi challenge there is a significant increase in Cbfα1/RUNX2 mRNA levels compared to the relative expression in normal VSMCs (t_0). **B**: Effect of lanthanum, gadolinium and calindol on Cbfα1/RUNX2 gene expression. Pretreatment with 100µM $LaCl_3$, 50µM $GdCl_3$ and 50nM calindol does not affect Cbfα1/RUNX2 levels four hours after high Pi challenge. Data were presented as mean ± SE of three different experiments (*p < 0.01).

We next studied the action of $LaCl_3$, $GdCl_3$ and calindol pretreatment on MGP mRNA levels modulation.

The time course shows that, after 5 days, the challenge with 5mM Pi results in a peak with a 35.2 ± 4.2 fold increase in MGP mRNA, compared to the relative expression in normal VSMCs (t_0 1.1 ± 0.3) (*p < 0.01) (Fig. 33A). The increase in high Pi treated cells is significant also if compared with MGP mRNA levels of control cells at day 5 of culture (20.7 ± 2.6 *vs* 35.2 ± 4.2 relative expression, ctr and 5mM Pi, respectively; * p < 0.01) (Fig. 33A).

$LaCl_3$ completely prevents the MGP mRNA increase induced by high Pi after 5 days of challenge (1.45 ± 0.02 *vs* 1.05 ± 0.05 of relative expression, 5mM Pi compared with 100µM $LaCl_3$; *p < 0.01) (Fig. 33B), whereas $GdCl_3$ does not affect high Pi-induced elevation in MGP levels (1.45 ± 0.02 *vs* 1.49 ± 0.05 of relative expression, 5mM Pi compared with 50µM $GdCl_3$; n.s.) (Fig. 33B). Considering the relative expression of MGP mRNA in control cells at day 5, we found that the pretreatment with 50nM calindol enhances the MGP gene expression significantly compared to the levels in high Pi-treated cells (1.45 ± 0.02 *vs* 1.97 ± 0.11 of relative expression, 5mM Pi compared with 50nM calindol; *p < 0.01) (Fig. 33B).

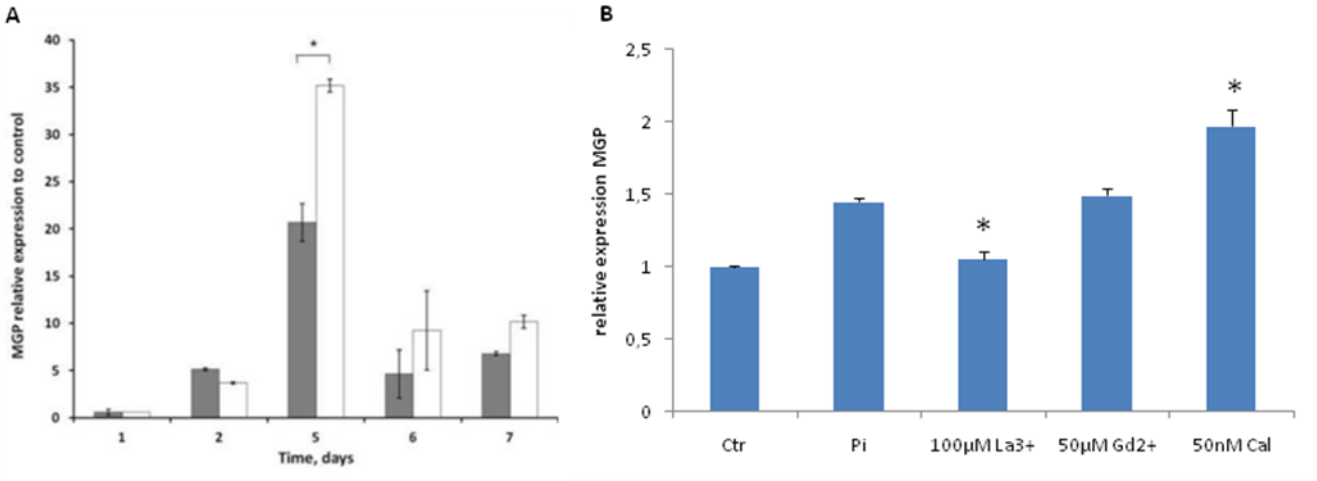

Figure 33: Effects of lanthanum, gadolinium and calindol on MGP gene expression

VSMCs were cultured in calcification medium and MGP mRNA expression was measured by RT-PCR. VSMCs were cultured for 5 days in calcification medium and MGP mRNA expression was measured.

A. Five days after high Pi challenge there is a significant increase in MGP mRNA levels compared to the relative expression in normal VSMCs (t_0) or control VSMCs at day 5 of culture. Relative expression of MGP mRNA in normal VSMCs (t_0) was used as reference value. **B.** Pretreatment with 100µM $LaCl_3$ maintained MGP mRNA levels as control sample, whereas 50µM $GdCl_3$ does not affect high Pi-induced increase in MGP mRNA. Pretreatment with 50nM calindol induces a significant increase in MGP mRNA levels 5 days after high Pi challenge. Data were presented as mean ± SE of three different experiments (*p < 0.01).

Osteonectin (SPARC) is a protein that, in our *in vitro* model, can be described as a marker of osteoblastic differentiation.

We studied its expression by immunohistochemistry technique, finding that control cells stain faintly and in less than 10% of cells (score 0, Fig. 34A), whereas high Pi-treated cells stain positive in 50-60% of cells (score 3, Fig. 34B). $LaCl_3$ and $GdCl_3$ have a different effect on SPARC protein expression. In fact, $LaCl_3$ almost completely prevents the high Pi-induced increase of stained positive cells (20%, score 1, Fig. 34C) and, on the contrary, cells treated with $GdCl_3$ stain positive in 60% of cells (score 3, Fig, 34D).

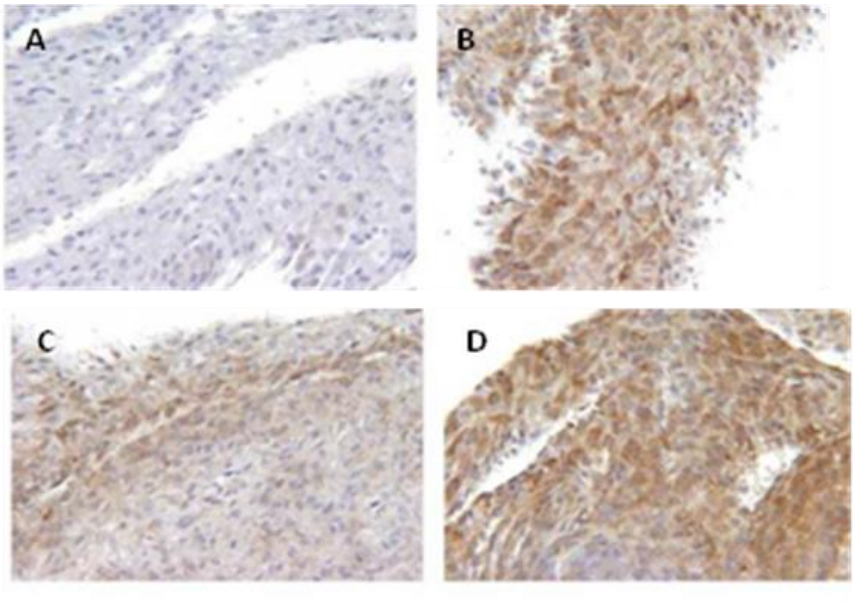

Figure 34: Effects of lanthanum and gadolinium on osteonectin expression

VSMCs were cultured for 8 days in calcification medium (**A**), with 5mM Pi (**B**), in presence of 100µM lanthanum (**C**) or 50µM gadolinium (**D**). A faint positivity was observed in osteonectin immunostaining in control sample (**A**); a more intense staining was observed after treatment with high Pi (**B**); a pattern similar to control was detected after pretreatment with lanthanum (**C**) whereas a pattern similar to high Pi-treated sample was detected after gadolinium pretreatment (**D**). Representative result of one of two different experiments.

6.3.3 Effect of $LaCl_3$ and $GdCl_3$ on the modulation of VSMC anti-apoptotic Axl protein expression

In our model, challenge with 5mM Pi for 8 days results in a decreased expression of the anti-apoptotic protein Axl as shown in figure 35 (113.2 ± 2.8% *vs* 94.2 ± 1.5% of intensity area, control compared to 5mM Pi; *$p < 0.01$). Interestingly, both 50µM and 100µM $LaCl_3$ preserve the expression of Axl protein to levels similar to control and significantly higher compared with high Pi-treated cells (94.2 ± 1.5% *vs* 104.1 ± 1.3% and 108.4 ± 2.1% of intensity area,

5mM Pi compared to 50µM $LaCl_3$ and 100µM $LaCl_3$, respectively; *p < 0.01) (Fig. 35A). On the contrary, 50µM $GdCl_3$ and 50nM calindol fail to restore Axl expression, without affecting the high Pi effect on this protein expression (94.2 ± 1.5% *vs* 94.7 ± 2.7% of intensity area, 5mM Pi compared to 50µM $GdCl_3$ (Fig. 35A) and 82.2 ± 4.9% *vs* 91.1 ± 5.1% of intensity area, 5mM Pi compared to 50nM calindol (Fig. 35B); n.s.).

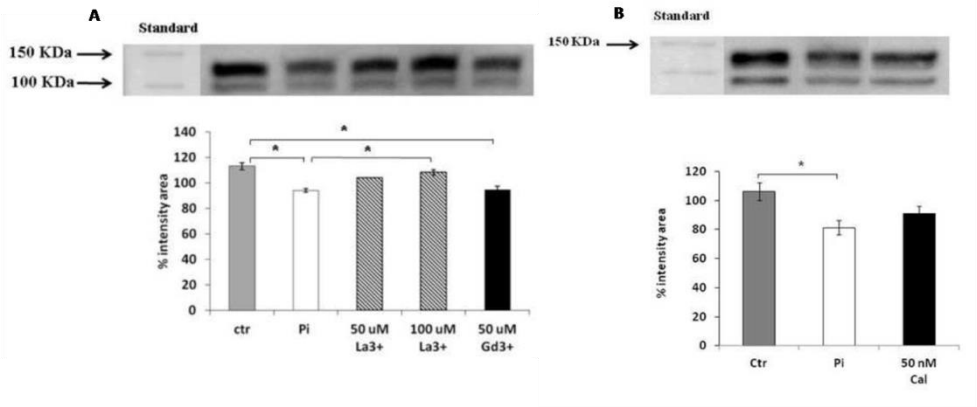

Figure 35: Effects of lanthanum, gadolinium and calindol on Axl expression
VSMCs were cultured for 8 days in calcification medium with lanthanum, gadolinium or calindol and challenged with 5mM Pi. Axl protein expression was detected by immunoblotting analysis. **A. Lane 1**: Control VSMCs; **lane 2**: 5mM Pi; **lane 3**: 50µM $LaCl_3$; **lane 4**: 100µM $LaCl_3$; **lane 5**: 50µM $GdCl_3$. **B. Lane 1**: Control VSMCs (106.2 ± 26.1%); **lane 2**: 5mM Pi; **lane 3**: 50nM calindol. Analysis of the intensity of the bands is shown. Lanthanum prevents, whereas gadolinium and calindol do not affect the high Pi-induced Axl down-regulation. Representative bands of one of three different experiments. Data were presented as mean ± SE of three different experiments (*p < 0.01).

7. DISCUSSION

Vascular calcification is associated with significant cardiovascular morbidity and mortality in patients with chronic kidney disease. Factors unique to CKD patients, such as hyperphosphatemia, predispose these subjects to early and progressive vascular calcium deposition. Hyperphosphatemia appears to be involved in a number of mechanisms that trigger and cause the progression of vascular calcification, including transition of vascular smooth muscle cells from a contractile to an osteochondrogenic phenotype, induction of apoptosis in VSMC and inhibition of autophagy.

In light of these pathological events high Pi mediated, it is important to limit vascular calcification in CKD patients through pharmacological therapy and dialysis, in order to control phosphate levels.

It is, also, important to elucidate which are the molecular events that trigger VSMCs to deposit calcium in the tunica media of vessels, and which are the promoter and inhibitor proteins involved in this pathological process in the attempt to design drugs to down-regulate the formers and to up-regulate the latters, with the final intent that control this pathological process.

Moreover, the knowledge of cellular and molecular events that lead to the pathogenesis of vascular calcification is useful to avoid the administration of therapy that, in any way, could interfere with the activity of proteins involved in these pathways and, consequently, made the CKD patients clinical situation worse.

7.1 MODULATION OF OSTEONECTIN EXPRESSION IN VASCULAR CALCIFICATION PATHOGENESIS

Osteoblastic transformation of VSMCs is one of the most important processes involved in vascular calcification, a multifactorial pathological process that results from an imbalance between the promoters and inhibitors of mineralization.

In our study, we decided to investigate the role of osteonectin, a major non-collagenous protein of bone matrix, in vascular calcification. Nevertheless there are some controversial results about its modulation during the process of vascular calcification, we have evidence that, in our model, it has a pro-calcificant role.

First of all, we investigated the time course expression of osteonectin in VSMCs challenged with 5 mM Pi in the attempt to understand its modulation with the progression of vascular calcification. We discovered that both control and high Pi-treated VSMCs are positive for osteonectin, with an up-regulation in cells treated with high-Pi compared to control. In high Pi-challenged VSMCs, the maximal osteonectin expression is at day 7. Its subcellular localization changes during the time course: indeed, until day 4, osteonectin is expressed only at a perinuclear level, to be expressed in the cytoplasm starting from day 7, where it is, presumably, destined for secretion.

To better understand if osteonectin is involved in the calcification process, we decided to correlate its time course expression with the progression of calcium deposition. At day 7, when osteonectin has a maximal expression, Von Kossa staining shows a massive calcium deposition in VSMCs cultivated in calcification medium.

Until now, we have evidence that osteonectin expression is induced in calcificant conditions, but we can not infer any conclusion about its role in VSMCs. Young et al demonstrated that osteonectin can regulate cell proliferation (135), so, to better understand the regulation of this protein in our model, we decided to study the correlation between its expression and cell proliferation. We investigated the time course expression of the proliferative nuclear marker Ki-67, demonstrating that, in calcific VSMCs, the maximal peak for Ki-67 is at day 4. The maximal expression of osteonectin and Ki-67 do not occur at the same time point, so, probably, the main role of osteonectin in our model is not the regulation of cell proliferation.

Then, since the agreement between the results of calcium deposition and osteonectin expression, we decided to elucidate the role of osteonectin in high Pi-induced calcification.

So, we investigated the time-course expression of Cbfα1/RUNX-2, the master gene involved in bone differentiation and in VSMCs osteoblastic transformation in calcific conditions. We found a maximal expression of Cbfα1/RUNX-2 at day 7, just like osteonectin. So, we started to suppose that osteonectin expression could be correlated with vascular calcification. Then we tried to associate its expression with another factor that we have previously demonstrated to be involved in the promotion of the mineralization process *in vitro*: ascorbic acid (AA). Therefore, we decided to study the modulation of osteonectin in VSMCs challenged with 5 mM Pi in presence and absence of AA, to understand if its regulation could be correlated with this cofactor of calcium deposition *in vitro*. The results show that AA modulates osteonectin expression: at day 7, the high Pi-induced calcification in absence of AA decreases of 60.8% compared to the high Pi-induced calcification in presence of AA. The positive association between Cbfα1/RUNX-2 elevation and AA presence with osteonectin expression supports a role for this protein in the high Pi-induced calcification of VSMCs.

The observation that osteonectin expression was induced in VSMCs *in vitro* in calcific conditions suggested that it could be involved in regulating the osteo/chondrocytic transition of VSMCs *in vivo*, so we decided to study its expression *ex-vivo* in fetal, adult non calcific and adult calcific atherosclerotic vessels. For the first time in our knowledge, we show a high expression of osteonectin in fetal VSMCs that is consistent with its involvement in processes such as tissue renewal, remodeling, and embryonic development. The localization of osteonectin in the active proliferating fetal VSMC indicates a potential role of this protein in mitosis regulation during development. Moreover, as expected, in adult specimens osteonectin is constitutively present with a faint staining that increases as calcification progresses, showing osteonectin expression in the residual VSMCs, which become displaced by fibrous tissue as the atherosclerotic plaque develops. Our findings are in agreement with the results by Dhore et al (45), considering osteonectin as a potential promoter of vascular calcification, but they are in contrast with those by other groups, which consider osteonectin as an inhibitory protein, down-regulated during arterial calcification (52-54).

In conclusion, although there are some controversial points of view about the modulation of osteonectin during the process of vascular calcification, our *in vitro* results allow us to

suppose that this protein could be involved in VSMC osteoblastic transformation, first of all because its maximal expression, in calcific conditions, is at the same time point in which there is the maximal osteoblastic transformation, and, then, because it is modulated in response to a cofactor of *in vitro* calcium deposition as ascorbic acid.

Our *ex vivo* data on human arteries support our *in vitro* data validating the hypothesis that the increase in osteonectin levels in sites of calcium deposition actively participates to the calcification process. These data may be of potential interest for better elucidate the pathogenesis of accelerated atherosclerosis in uremic patients that suffer of both hyperphosphatemia and vascular calcification.

7.2 "WASH OUT" EFFECTS ON VASCULAR CALCIFICATION

Known the abilities of VSMCs to remodel their phenotypic characteristics in response to environment changes and known the key role of high phosphorous on vascular calcification and on osteoblastic differentiation of VSMCs, the main purpose of this *in vitro* studies was to investigate the mechanism of high Pi-induced vascular calcification: in particular, we analyzed the effect of temporary and repeated suspension of high Pi on the progression of vascular calcification (process that we called "Wash Out") in order to develop an experimental model that can simulate *in vitro* the haemodialysis treatment. Our purpose was to investigate the high Pi impact on vascular calcification and the potential benefits that "Wash Out" could afford in terms of prevention of calcium deposition.

The present study demonstrates that "Wash Out" treatment partially protects vascular smooth muscle cells from high-Pi-induced calcification: it is a very innovative discovery in the field of vascular calcification, because this is the first time that the effect of short term high-Pi suspension on VSMC calcium deposition demonstrates an efficacy *in vitro*, without the use of any pharmacological treatment.

First of all, we studied the "Wash Out" effect on VSMC calcium deposition and we demonstrated not only that there is a beneficial effect on vascular calcification as a consequence of short time high Pi suspension, but, also, that the inhibition of calcium deposition depends on the duration of this suspension: longer is the time of high Pi suspension, higher is the inhibition of calcium deposition compared to cells constantly exposed to high Pi. In particular, in cells that received the "Wash Out" treatment for 4 hours every time medium was replaced there is a high and significant inhibition of calcium deposition, about 35-40%, compared to cells continuously challenged with high Pi. It is surprising that the suspension of high-Pi treatment for only 8 hours during 7 days of calcification can induce a so consistent and significant inhibition of calcium deposition. So, although high Pi is a very strong trigger of VSMC calcification, these results suggest that it is enough a short time high Pi suspension to delay VSMC calcium deposition.

Then, we investigated if the number of high Pi suspensions could have an effect on calcium deposition. In 7 day experiments, high Pi was suspended twice for only 8 hours overall, whereas in 10 day experiments three times for 12 hours overall: we don't find relevant differences in terms of "Wash Out" protective effects on calcium deposition, being these

percentages of inhibition comparable (35.78% and 45.16% of inhibition, day 7 and day 10 of calcification, respectively). In 10 days experiments, cells were cultured in the presence of 15% fetal bovine serum to allow them to grow healthily and to avoid apoptosis and necrosis events, because dead cells are foci for calcium deposition. Therefore, when the calcification model is set up, the "Wash Out" protective effect is kept and it is independent from the number of times of high Pi suspension, but is clearly dependent on the duration of the pro-calcific stimulus suspension.

To confirm that the final protective effect of "Wash Out" treatment is really due to short high Pi suspension, we decided to culture cells in continuously presence of high Pi and, rather than suspend the challenge for 4 hours every time medium was replaced, we added Phosphonoformic acid (PFA) for the same "Wash Out" period, in order to mimic the total absence of phosphorous in our *in vitro* model for short periods, and we analyzed the final effect on calcium deposition. It is important to mention that in our *in vitro* model inorganic phosphate can exist in 2 forms, free soluble Pi and as insoluble hydroxyapatite crystals. PFA, besides to be a competitive inhibitor of sodium-dependent phosphate co-transporter (Pit-1), able to induce a dose-dependent inhibition of VSMCs phosphate uptake, it is, also, an inhibitor of hydroxyapatite crystal formation, preventing nucleation in a similar way to pyrophosphate and bisphosphonates (87, 119). Therefore, in the presence of PFA, phosphorous can not enter in VSMCs and can not precipitate as crystals. In our *in vitro* model, short time PFA treatment confirms the "Wash Out" results in terms of inhibition of vascular calcification: in cells treated with PFA for the same "Wash Out" period every time medium was replaced there is an inhibition of calcium deposition of 42% compared to cells constantly exposed to high Pi levels during 7 days calcification process, and this protective effect is comparable to those resulting from the "Wash Out" treatment.

This supplementary study confirms that the delay on the progression of VSMC calcium deposition in "Wash Out" model is really due to the temporary and repeated absence of high Pi, supporting the relevant role of high Pi on vascular calcification.

As already mentioned, extracellular Pi enters into the intracellular compartment via a sodium-dependent phosphate co-transporter (Pit-1)-mediated pathway. This influx increases during hyperphosphatemia and it leads to the accumulation of intracellular phosphate. By pathways that have not yet been fully elucidated, the increased intracellular phosphate acts

as a promoter signal for osteogenic gene expression (Cbfα-1 and downstream targets osteopontin and osteocalcin) and as a suppressor of smooth muscle cells specific gene expression, resulting in increased secretion of mineral-nucleating molecules (matrix vesicles, calcium-binding proteins, alkaline phosphatase, and collagen-rich extracellular matrix) (75). Given the importance of Pi influx in VSMCs to promote vascular calcification, Pit-1 has a central role in the pathogenesis of calcification: numerous studies demonstrated that the suppression of endogenous sodium-dependent phosphate co-transporter in VSMCs by small interfering RNAs (siRNAs) prevents high-Pi-induced VSMC calcification *in vitro* (84), that the Na/Pi co-transporter inhibitor PFA prevents calcification and osteogene expression in the presence of hyperphosphatemic concentrations (75, 87) and that Pit-1 expression in VSMCs increases under some calcifying conditions (75, 124, 136).

As already mentioned, inorganic phosphate, besides to be a soluble signaling molecule, can precipitate as insoluble hydroxyapatite crystals: it has been recently reported that hydroxyapatite crystals alone are able to induce the process of VSMC osteogenic transformation. Sage et al. demonstrated that purified synthetic hydroxyapatite nanocrystals and isolated hyperphosphatemia-induced nanocrystals, up-regulate the expression of Bmp-2 (bone morphogenetic protein-2) and osteopontin genes in VSMCs *in vitro* (25). It is possible that nanocrystals directly affect DNA transcription complexes, as they have been reported to enter the nucleus and interact with histones (137). However, the molecular mechanisms or pathways by which these nanocrystals act on gene expression are not fully elucidated. The findings of Sage et al. support the view proposed by Shanahan (138) that vascular calcium crystals are far from inert and may promote the pro-inflammatory, pro-osteogenic environment associated with vascular calcification. Some studies demonstrated that nanocrystals have pleiotropic effects, including the induction of inflammatory cytokines in macrophages, mitogenesis and matrix metalloproteinases in osteoblasts and fibroblasts, autophagy, and, under serum-free conditions, the induction of apoptosis in VSMCs (138).

In our experimental conditions, we add 5 mM Pi to cells to induce vascular calcification, and this high concentration is over the solubility of Ca x Pi product: therefore, Pi can not be totally in solution, but it also precipitates as insoluble crystals together with other cations like Ca^{++}.

First of all, we measured the concentration of free Pi and of Pi complexed in hydroxyapatite crystals, and we found that it was the same, about 2.5mM.

Since inorganic phosphate can act in these two forms, one of the aim of our experiments was to elucidate what is the single potential contribute of free Pi and hydroxyapatite crystals during the 4 hours of repeated high Pi suspension on 7 days calcium deposition: in other words, we decided to plan different experimental strategies to investigate the effect of the absence of free Pi or hydroxyapatite crystals during the "Wash Out" period on the delay of the progression of VSMC calcium deposition.

Therefore, as a first experimental approach, we decided to study the modulation of calcium deposition in response to the blockage of Pi influx in VSMCs through Pit-1 during the repeated and short time high Pi suspensions, in order to elucidate only the contribution of hydroxyapatite crystals during the "Wash Out" period on calcium deposition: in this way, we can demonstrate the relevance of Pi influx blockage in VSMCs on the prevention of high Pi-induced vascular calcification in our *in vitro* model. To answer to this question, we planned three different experimental strategies: in the former, we temporary blocked Pi entry in VSMCs by Pit-1 through the elimination of the Na^+ gradient, that is necessary to allow Pi influx in cells; in the second, we tried to address PFA protective action only on the blockage of Pi entry in VSMCs, trying to eliminate the contribute of PFA effect on hydroxyapatite crystals formation; in the latter, we directly investigated the single contribute of hydroxyapatite crystals on calcium deposition during the "Wash Out" period.

For the first approach, we decided to replace calcification medium with a "choline-saline solution" for a short period every time medium was replaced in continuously presence of high-Pi. In this set of experiments, some cells were treated for short periods with "choline-saline solution", in which sodium was replaced with choline, a molecule able to maintain cellular osmotic pressure, but too large to be co-transported with Pi into VSMCs through Pit-1. Treating the cells with this saline solution for 4 hours every time medium was replaced resulted in cell death: probably because the cells were in absence of serum, amino acids and vitamins, that are essential components of calcification medium and that are, probably, necessary to limit the toxic effect of high Pi. So, we decreased the period of this temporary treatment from 4 to 2 hours. Replacing sodium with choline abolishes phosphate uptake by VSMCs: with the short time blockage of Pi influx in VSMCs every time medium was replaced,

there is a 18% of inhibition of vascular calcification compared to cells constantly challenged with high-Pi in the presence of a Na^+ gradient.

For the second approach, we used a pharmacological treatment: in particular, we addressed PFA action only on the blockage of Pit-1, and not on the inhibition of hydroxyapatite crystals formation by pre-incubating calcific medium O/N at 37°C to allow the formation of hydroxyapatite crystal. Even with this experimental strategy, we confirmed that the consequence of short and repeated Pi influx suspensions in VSMCs is an inhibition of calcium deposition of 37% compared to cells constantly challenged with high Pi for 7 days, and this inhibition is about the same resulting from "Wash Out" treatment. It means that the only presence of hydroxyapatite crystals for repeated short period leads to a final calcium deposition comparable to those resulting from "Wash Out" condition.

Finally, even with the third approach, we demonstrated that in absence of free Pi and with the only presence of hydroxyapatite crystals for the "Wash Out" period, calcium deposition results inhibited for 37% compared to cells constantly challenged with high Pi for 7 days.

These results lead us at the conclusion that the absence of free Pi and, therefore, the only presence of hydroxyapatite crystals for repeated short period leads to a inhibition of calcium deposition comparable to those resulting from "Wash Out" condition.

Therefore, as a second experimental approach, we investigated the other side of the coin, that is the modulation of calcium deposition in response to the only presence of free Pi during the repeated and short time high Pi suspensions: in this way, we can deduce the relevance of the short period absence of hydroxyapatite crystals on the prevention of high Pi-induced calcium deposition in our *in vitro* model. The consequence of short and repeated suspensions of Ca-P crystals during the "Wash Out" period is an inhibition of calcium deposition of 37% compared to cells constantly challenged with high Pi for 7 days, about the same resulting from "Wash Out" treatment and from the only presence of hydroxyapatite crystals during the "Wash Out" period.

Therefore, the results of these different experimental strategies demonstrate that both the temporary absence of Pi entry in VSMCs and the temporary lacking of Ca-P crystals presence during 7 days of high Pi challenge lead to a unique and unquestionable result, that is the partial inhibition of calcium deposition compared to cells continuously high Pi-challenged: interestingly, the percentages of inhibition resulting from the temporary absence of one of

the two Pi components are the same and they are comparable to the percentage of inhibition obtained from the total absence of high Pi during the “Wash Out” period.

So, we can infer that it is not relevant challenging VSMCs either with (free Pi or Ca-P crystals) to partially prevent VSMC calcification during “Wash Out” period, but what is important is temporarily decrease Pi challenge under a so called trigger threshold, even for short period, during the process of calcification to obtain a substantial inhibition of calcium deposition after 7 days of high Pi conditions. When cells are temporarily challenged only with free Pi or hydroxyapatite crystals, they are exposed to an under threshold phosphorous burden, that is about the half compared to the total high Pi used to challenge cells during the calcification process (2.5-3mM *vs* 5mM), and this condition prevents VSMC calcium deposition in the same extend as the total absence of phosphate in the “Wash Out” experiments. The relevance of the Pi threshold on the final event of calcium deposition was demonstrated even in the set of experiments that correlates the duration of the “Wash Out” period with the prevention of calcium deposition, where longer was the “Wash Out” period, longer was the time in which Pi levels were under the threshold, and, as a result, higher was the prevention of calcium deposition.

These results confirm that the mechanism of action of Pi in the process of VSMC calcification is a so called “on-off” mechanism: when Pi levels are under the threshold, that in our *in vitro* model is about 4.5-5mM, the load of phosphorous that cells sense is not sufficient to trigger all a series of intracellular events that lead them to deposit calcium; however, it is sufficient a little bit ovestep of the threshold (to begin from 5mM Pi) to suddenly “switch on” these events, with the final effect of VSMC calcium deposition. Given that in wash out experiments, in the “threshold” periods cells freeze the processes that finally lead to calcium deposition because the action of Pi is “off”, the final effect is a surprising and significant reduction of calcium deposition compared to cells constantly challenge with high Pi for 7 days (about 40% of of calcium deposition inhibition).

These experimental observations suggest how is important to limit high Pi contact with VSMCs to delay the progression of vascular calcification. Our *in vitro* results demonstrate that the simply suspension of the pro-calcific factor “high-Pi” for 8 hours during 7 days high Pi-treated VSMCs has a very significant impact on the inhibition of calcium deposition. This *in vitro* discovery can suggest that it should be opportune to increment the duration of

haemodyalisis cycles in CKD patients, and so, to increment the duration of the under threshold condition, in the attempt to control as more as possible phosphatemia. It was already demonstrated that nocturne haemodyalisis is the gold standard to decrease phosphoremia in CKD patients, since they do not need to assume any phosphate binders pharmacological therapy. Since longer is the duration of haemodyalisis, longer is the time in which cells are exposed to an under threshold circulating Pi level, we could suppose that the result of an increment of the time in which VSMCs are not exposed to high Pi could be the decrease of vascular calcification (139).

The final effect of "Wash Out" is the reduction of calcium deposition, but we don't know what are the mechanisms involved. Since VSMC calcification is a multifactorial process that arises from the modulation of different biological pathways, in the "Wash Out" experiments we decided to focus our attention on the study of the potential mechanisms involved in this protective process, as the modulation of VSMC lineage markers, apoptosis and autophagy.

First of all, we investigated the effect of "Wash Out" treatment on the modulation of vascular smooth muscle cell markers expression. Under normal conditions, VSMCs express smooth muscle lineage markers, including α-actin and SM22α. We demonstrated that treatment with high Pi causes a loss of SM lineage markers, but their expression was almost fully recovered with a 4 hours suspension of 5mM Pi every time medium was replaced. The percentage of inhibition of α-actin expression by 5mM Pi compared to control condition is 37.5%, while Pi-4h caused a down-regulation equal to 12.0% compared to control condition; in 4h high Pi treatment suspension condition there is a recovery equal to 68% of α-actin expression. The same percentage of recovery is achieved also after "Wash Out" treatment for SM22α expression.

Therefore, we demonstrated that at least one mechanism by which "Wash Out" exerts a protective effect on calcium deposition is the prevention of high Pi-induced down-regulation of VSMC markers, with the preservation of a muscular cell phenotype and the inhibition of their-simil-osteoblastic transformation. It is important that VSMCs can keep their contractile phenotype to contrast cardiovascular events. However, it could be very interesting to study "Wash Out" effects on the modulation of osteoblastic markers in next experiments.

It's not clear if the protective effect of high Pi suspension involves homogeneously all cells, that become able to partially arrest or decelerate calcium deposition or osteoblastic

transformation, or if it is the result of the response of only a subgroup of cells. Being calcium deposition a focal process *in vitro*, if the transformation corresponds to calcification foci, we can hypothesize that some cells do not transform themselves, some arrest their transformation process at a middle level, and others complete their osteoblastic transformation.

After the study of the potential "Wash Out" effects on VSMC markers modulation, we investigated whether high Pi suspension could be involved in the prevention of cell apoptosis. Since it is known that apoptosis is involved in VSMC calcification and that apoptotic bodies derived from VSMCs may act as nucleating structures for calcium crystal formation, we decided to investigate if "Wash Out" treatment could prevent this pro-calcificant event: in particular, we focused our attention on the modulation of DNA fragmentation, that it is the final event of cell apoptosis. We demonstrated that "Wash Out" prevents high Pi-induced VSMC apoptosis by the decrease of DNA fragmentation: in cells that received a 4 hours high Pi suspension every time medium was replaced there is a partially DNA fragmentation reduction compared with cells continuously exposed to high Pi. This "Wash Out" anti-apoptotic effects is maintained during the progression of vascular calcification, until 11 days of culture in calcification medium, with a major protection at day 11 compared to day 7 (39.6% and 25.0%, respectively), probably because these cells received a supplementary "Wash Out".

Since "Wash Out" modulates DNA fragmentation, we investigated a potential anti-apoptotic pathway that has been demonstrated to be involved in high Pi-mediated apoptosis to find out whether it could be modulated by "Wash Out" treatment: the Axl-Gas6 pathway. To maintain cell survival, the binding of Gas6 to its cognate receptor Axl induces phosphatidylinositol 3-OH kinase (PI3K) activation and subsequent Akt activation, showing sequential events of the Gas6/Axl/PI3K/Akt signal (35). In our experimental model, the downregulation of Axl associates with calcification, whereas the maintenance of its normal levels with the prevention of VSMC calcification; neverthless, despite the anti apoptotic action of "Wash Out" on DNA fragmentation to prevent VSMC apoptosis, it seems that the temporary high Pi suspension does not affect the modulation of Axl and this pathway in our *in vitro* model. However, it is known that high Pi can affect apoptosis by

other pathways, for example it can modulate the caspase 3 and 9 activity and can modulate the activity of mithocondrial citocrome c.

Finally, we started to investigate the “Wash Out” effect on a potential mechanism that seems to be involved in vascular mineralization: autophagy. Autophagy is a dynamic and highly regulated process of self-digestion responsible for cell survival and reaction to oxidative stress. Dai et al. recently demonstrated that calcium deposition in VSMCs increases during inhibition of autophagy (116), defining autophagy in the high Pi model as a protective mechanism with respect to calcium deposition. So, we decided to use electron microscopy as a first qualitative approach to elucidate whether high Pi suspension could modulate this anti-calcific process. Electron microscopy is the most appropriate technique to study fine ultrastructural modifications that accompany cell death and autophagy. Our results show an increment of autophagic structures in VSMCs that received “Wash Out” treatment compared to cells continuously exposed to high Pi. Electron microscopy images show that “Wash Out” treated cells try to preserve their vitality by the isolation of damaged organelles in double-membrane vesicles (autophagosomes) that will be, probably, targeted to lysosomes to be digested in intracellular structures called autophagolysosomes in order to create new nutrients for survival and to selectively remove damaged mitochondria, in the attempt to prevent the activation of apoptotic pathways that lead, as a last stage, to calcium deposition. Moreover, whereas VSMCs that received “Wash Out” treatment show increased autophagosomes, cells continuously exposed to high Pi are rich in calcific mitochondria, that are at a higher degenerative stadium than “Wash Out” mithocondria. The high Pi-treated cells implement the expulsion of calcific organelles, as shown by the electron microscopy images, probably because in these cells autophagy can not afford to eliminate all the damaged mithocondria, and some of them calcify inside the cells before the cell can, in any way, eliminate them. In cells continuously challenged with high Pi, mithocondrial calcification certainly could lead to organelles dysfunction, like the decrease of mitochondrial membrane potential and ATP production and the concurrently increased production of reactive oxygen species. In VSMCs that received “Wash Out” treatment, mitochondria are less calcified and more structurally preserved, and it could be one of the reasons that lead to a decrease in VSMC apoptosis. So, in next experiments we would like to focalize the study of apoptosis on mitochondrial-dependent apoptotic events, including

release of cytochrome c from the mitochondria into the cytosol and subsequent activation of caspase-9 and -3, proteins involved in DNA fragmentation apoptotic pathway.

Although electron microscopy can provide an impressive amount of ultrastructural information, the visual inspection of electron microphotographs should always be complemented by a quantitative approach. So, to support these qualitative results, we tried to elucidate the autophagy process by the western blot analysis, and we investigated the modulation of an autophagocitic marker, LC3-II (Light Chain 3-II), to ensure the involvement of this process in VSMC calcification. Analyzing this marker, we demonstrated that cells continuously challenged with high Pi express higher levels of the autophagic marker compared to control and "Wash Out"-treated cells. Since autophagy is a dynamic cell process, the accumulation of autophagosomes is not always indicative of autophagy induction, and may represent either the increased generation of autophagosomes and/or block in autophagosomal maturation and the completion of the autophagy pathway. So, we analyzed the expression of this marker monitoring "autofagic flux" by western blot assay: we treated cells with chloroquine, an agent that impairs lysosomal acidification and stops autophagosomes degradation, with the consequent block of the basal LC3-II turnover and the accumulation of this marker. Results show that the difference in LC3-II levels in the presence and absence of chloroquine is larger in control and in "Wash Out"-treated cells than in high Pi-challenged VSMCs, indicating an increment of the autophagic flux in the former conditions. Western blot analysis leads us to suppose that, probably, cells continuously challenged with high Pi activate autophagy process, because they are exposed to stress conditions: so they "switch on" this protective mechanism in the attempt to survival, and it is indicated by high LC 3-II levels in absence of chloroquine. However, they aren't able to complete the process, and it is demonstrated by "autofagic flux "experiment, where we found similar levels of LC3-II in the presence and absence of chloroquine: this result suggests that in high stress condition, the completion of the autophagy pathway is suppressed, resulting in decreased autolysosomal degradation of LC 3-II. Probably, high Pi condition are too toxic to maintain this life-and-death struggle, and when they are too stressed to try to maintain their survival, they surrend themselves and they go towards apoptosis. Interestingly, in "Wash Out" condition there is an increment of the autophagic process probably because cells are in better health compared to cells continuously

challenged with high Pi: in these repeated short time periods in which Pi action is “switch off”, they try to protect themselves further activating autophagy in order to eliminate the damaged intracellular structures to survive.

In conclusion, hyperphosphatemia appears to be involved in a number of mechanisms that trigger and cause VSMC pathological calcium deposition: when Pi levels are over a threshold, that in our *in vitro* model coincide with 5mM, cells are stimulated to “switch on” all a series of intracellular events Pi-dependent, including their transformation from a contractile to an osteochondrogenic phenotype, induction of apoptosis and decreasing in autophagic protective process, that, all together, lead them to deposit calcium at an extracellular level.
As we demonstrated in our *in vitro* model, when we challenged VSMC with high Pi, this inorganic ion is present in two different forms, free Pi and as hydroxyapatite, respectively: both of these Pi types act synergically on cells leading to VSMC calcification.
To obtain the pathological VSMC calcium deposition, what is really important is the presence of high Pi level that overstep the threshold. Effectively, when VSMCs are continuously challenged with over threshold Pi levels, Pi action is constantly “switch on” and cells respond to this constant pro-calcific stimulus triggering and perpetuating their pathological calcium deposition. On the contrary, both in “Wash Out” model, where VSMC high Pi treatment is temporarily suspended, and treating cells for short periods with Pi levels that are under threshold, it is clearly evident the surprising benefit on the delay of the progression of vascular calcification. Surprising, all the high Pi-modulated intracellular processes that trigger VSMC calcium deposition (down-regulation of VSMC markers and autophagy, and up-regulation of VSMC apoptosis) are delayed because cells are challenged with “under threshold” Pi levels only for short periods compared to the 7-11 days of high Pi conditions, but these repeated and short periods are sufficient to temporarily freeze VSMC transformation process and apoptosis with a substantial and significant inhibition of the final calcium deposition.
In light of the high Pi pathological action on VSMC calcification process and of the protective effects of the *in vitro* “Wash Out” mechanism, it is of primary importance to decrease the time of contact between arteries and high Pi in dialysis patients in the attempt to reduce VSMC calcification and the consequent risk of cardiovascular events: for example, it will be

opportune to prolong haemodialysis cycles to limit the time in which VSMC are exposed to an high Pi loading.

Understanding the role of phosphate and improving the management of hyperphosphatemia is of relevant importance. Despite the significant amount of knowledge in the field, important questions remain to be answered. Whether vascular calcification can be prevented or reversed with strategies aimed at maintaining phosphate homeostasis presently is not fully elucidated.

7.3 EFFECT AND MECHANISMS OF ACTION OF LANTHANUM CHLORIDE ($LaCl_3$), GADOLINIUM CHLORIDE ($GdCl_3$), AND CALINDOL ON THE DELAY OF THE PROGRESSION OF HIGH Pi-INDUCED VASCULAR CALCIFICATION

Vascular calcification represents a major cardiovascular risk factor in chronic kidney disease patients and high phosphate levels are strongly associated with vascular calcification in this population. In dialysis patients, hyperphosphatemia can be reduced by the control of oral Pi intake, an adequate dialysis treatment, and the use of intestinal Pi-binders. In particular, the use of calcium-free Pi-binders, such as lanthanum carbonate, appears to be an essential part of the treatment to control Pi overload, reducing the risk of hypercalcemia and vascular calcification (140).

Moreover, high serum phosphate levels associate with secondary hyperparathyroidism (SHPT), and, to control this complication, dialysis patients are treated with active vitamin D and/or the calcimimetic cinacalcet (141). Beyond SHPT control, calcimimetics have been investigated as new potential tools to control vascular calcification. In animals affected by renal failure, Lopez et al. demonstrated that treatment of SHPT with active vitamin D, but not with calcimimetics, was associated with the development of vascular calcification (142). Recently, it has been also reported a reduced expression of the calcium sensing receptor (CaSR) in calcified vessels, with a prevention of arterial CaSR downregulation by calcimimetics (143, 144).

Thus, the aim of this pharmacological study was to investigate the mechanisms of action of the Pi-binder lanthanum chloride, compared to gadolinium chloride, and of the calcimimetic calindol on the progression of high Pi-induced calcium deposition through their direct effects on VSMCs *in vitro*.

First of all, we demonstrated that lanthanum chloride, gadolinium chloride and calindol prevent VSMC calcium deposition after 7 days of high Pi challenge: indeed, calicium deposition was concentration-dependently inhibited by addition of $LaCl_3$, $GdCl_3$ and calindol, with a maximal inhibitory effect obtained with 100µM (39.80% of inhibition), 50µM (56.40% of inhibition) and 100nM (64.0% of inhibition), respectively. Moreover, we elucidated wheather the protective effect of $LaCl_3$, $GdCl_3$ was persistent with the progression of calcium deposition, 15 days after challenge VSMCs with high Pi: our results demonstrate that when the calcification model is set up, the protective effect of lanthanum and gadolinium is kept

(45.10% and 61.20% of inhibition, respectively), and it is independent by any chelating effect on phosphate, since Pi levels in the culture medium result unchanged after $LaCl_3$ and $GdCl_3$ addition at the highest concentration (*data not shown*).

Subsequently, we investigated potential intracellular mechanisms of action of these three compounds that could be involved in the delay of the progression of high Pi-induced VSMC calcium deposition.

At the beginning, we studied the effect of these three pharmacological treatments on the modulation of VSMC lineage markers expression under high Pi conditions. Under normal conditions, VSMCs can express smooth muscle lineage markers that represent the contractile phenotype, including α-actin and SM22α. It has been intensively elucidated how treatment with high Pi causes a loss of smooth muscle lineage markers, preceding osteochondrogenic markers up-regulation, such as Cbfα-1/Runx2 and BMP-2, thus inducing VSMC phenotypic trans-differentiation. Potentially, this effect may, in part, explain the strong association between high serum Pi levels, increased ectopic calcification burden, and enhanced cardiovascular morbidity and mortality in CKD and dialysis patients (48, 81, 82).

We demonstrated that $LaCl_3$ prevents the high Pi-induced down-regulation of two VSMC markers, such as α-actin and SM22α . In contrast, $GdCl_3$ and calindol do not affect the reduction of the smooth muscle lineage markers induced by high Pi.

Thus, lanthanum, gadolinium and calindol reduce high Pi-induced calcium deposition in VSMCs, but only $LaCl_3$ appears to preserve the normal expression of vascular smooth muscle lineage markers. In this study, for the first time, we demonstrated that lanthanum reduces vascular calcification by preventing high Pi-induced down-regulation of VSMC lineage markers.

To further elucidate the molecular mechanisms by which these compounds prevent vascular calcium deposition, we next analyzed the gene expression of three proteins involved in vascular calcification, such as BMP-2, Cbfα1/RUNX2, MGP and the osteonectin protein expression (SPARC), all factors actively involved in the osteoblastic differentiation of VSMCs when incubated in high Pi media.

First, we observed a peak in BMP-2 mRNA compared to the relative expression in normal cells.

Intriguingly, $LaCl_3$, $GdCl_3$ and calindol effectively prevented the high Pi-induced up-regulation of BMP-2 mRNA expression. BMP-2 plays an important role in the regulation of bone formation, and more recently it has been described its potential role in regulating vascular calcification (124). In fact, BMP-2 induces osteoblastic differentiation and mineralization. Moreover, calcified human and murine atherosclerotic lesions express BMP-2, whereas treatment of bovine VSMCs with BMP-2 enhanced calcium deposition (41). Our studies confirm the important role of BMP-2 in regulation of vascular calcification, with the possibility to prevent osteoblastic differentiation by pretreatment with lanthanum, gadolinium and calindol.

Afterward, we confirmed that high Pi induces an increase on Cbfα1/RUNX-2 mRNA, but $LaCl_3$, $GdCl_3$ and calindol pretreatments are not able to down-regulate the expression of this gene deeply involved in osteoblastic differentiation in the vasculature. The fact that these pretreatments show any effect on preventing the high Pi-induced increased Cbfα1/RUNX-2 gene expression may be due to a mechanism affecting other pathways different from Cbfα1/RUNX-2, such as Msx2, osterix, and/or Sox9.

Our *in vitro* studies suggest that high Pi causes vascular calcification and induces osteoblastic VSMCs trans-differentiation by up-regulating both BMP2 and Cbfα1/RUNX-2 gene expression. $LaCl_3$, $GdCl_3$ and calindol appear to share a common downstream signalling pathway for prevention of vascular mineralization, reducing BMP-2 with no effects on Cbfα1/RUNX-2 gene expression. Similar data have been obtained in different experimental conditions by Speer et al. (145) in calcified arteries from MGP-/- mice, where Cbfα1/RUNX-2 mRNA expression was reduced compared to controls, whereas the osteochondrogenic transcription factor BMP2 was highly up-regulated.

Furthermore, we investigated the effects of $LaCl_3$, $GdCl_3$ and calindol pretreatment on Matrix Gla Protein (MGP) mRNA levels modulation. MGP is a member of the vitamin-K dependent protein family, which has a high affinity for calcified areas and normal media of blood vessels, and appears to inhibit locally calcium deposition. Its role in contrasting vascular calcification was suggested by the findings that MGP-/- mice develop extensive extra-skeletal calcification and severe bone disease. Interestingly, both CKD and haemodialysis patients have a different distribution of MGP gene polymorphism, with increased risk of cardiovascular events as compared to the normal population (146). Surprisingly, we found

that $LaCl_3$ completely prevents, $GdCl_3$ does not affect MGP mRNA increase induced by high Pi and calindol significantly enhances MGP gene expression compared to high Pi-treated VSMCs.

Our findings suggest that calcium sensing receptor (CaSR) may participate in MGP regulation in the vasculature. During the last decade, the CaSR expression in VSMCs has been debated (147). More recently, the existence of a functionally active CaSR in VSMCs has been well documented (148). Interestingly, Mendoza et al showed that up-regulation of MGP expression in bovine VSMCs by other calcimimetics, such as AMG 641, requires high concentration of calcium (149). On the contrary, our previous results demonstrate that the calcimimetic calindol prevents high Pi-induced vascular calcification independently by calcium concentration (150). Our *in vitro* studies provide a potential mechanism of how calcimimetics may prevent high Pi-induced vascular calcification. In fact, calindol prevents vascular calcification by delaying the expression of the pro-calcific gene BMP-2 and by up-regulating the high Pi-induced anti-calcific MGP gene expression. More studies are necessary to better elucidate the intracellular mechanism involved in MGP regulation by the calcimimetics in VSMCs.

Lanthanum delays the anti-calcific gene (MGP) expression probably due to the preservation of smooth muscle lineage markers and the prevention of VSMC transdifferentiation. In contrast, gadolinium does not modify the high Pi-induced elevation of MGP mRNA levels, supporting the notion that lanthanum and gadolinium might not share common regulatory mechanisms in the pathogenesis of vascular prevention.

Afterwards, we investigated osteonectin expression, described as a marker of osteoblastic differentiation (56). Also on osteonectin protein expression, $LaCl_3$ and $GdCl_3$ show different effects: similar to MGP, only lanthanum greatly prevents high-Pi-induced elevations of osteonectin protein levels, whereas gadolinium does not. Osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC) is a glycoprotein secreted by osteoblasts during bone formation, with high affinity for bone mineral calcium. Davies et al. observed that protein expression of osteonectin and MGP was higher in patients with systemic sclerosis. In particular, SPARC expression was significantly greater in both endothelial cells and fibroblasts of those patients with greater calcification (calcinosis) (151). The finding that only $LaCl_3$ prevents the increase in ostenectin levels, while $GdCl_3$ show no effects on SPARC

enhancement, confirms that these two compounds act with different mechanisms on vascular calcification.

Since apoptosis regulates vascular calcification *in vitro*, we next investigated the effects of $LaCl_3$, $GdCl_3$ and calindol on Axl expression, a membrane tyrosine kinase receptor actively involved in this process. Down-regulation of Axl has been associated with calcification, whereas the maintenance of its normal levels with prevention of both apoptosis and, thus, arterial mineralization (34, 153). In our model, high Pi decreases the expression of Axl. Interestingly, $LaCl_3$ preserves Axl protein expression to levels similar to controls. In contrast, $GdCl_3$ and calindol fail to prevent the high Pi-induced Axl down-regulation. Collett et al. have reported that Gas-6 and its receptor Axl play a key role in osteogenic differentiation of vascular pericytes in an autocrine manner (153). More recently, Son et al. demonstrated that both fluvastatin and pravastatin may reduce vascular calcification *in vitro* by inhibiting apoptosis, acting on gas6/Axl expression (35). In fact, statins significantly inhibited high Pi-induced apoptosis and calcification in a concentration-dependent manner in human VSMCs. In our studies we demonstrates that $LaCl_3$ prevents high Pi-induced Axl down-regulation. These findings indicate that the Axl-mediated survival pathway is a potential target of lanthanum effect to prevent vascular calcification.

Additionally, the actions of BMP-2 may increase apoptosis, so, if the cells pretreatment with lanthanum causes a reduction of expression of BMP-2 and an up-regulation of Axl, it is plausible that this down-regulation is correlated with the reconditioned expression of the anti-apoptotic protein Axl compared to cells challenged with high Pi and without pretreatment. For the other two pretreatments, that lead to a down-regulation of BMP-2 too, the anti-apoptotic pathway involved could be different from Gas6-Axl pathway.

These *in vitro* studies may suggest a potential role for lanthanum in preventing vascular calcification and osteoblastic differentiation. In the clinical setting, oral lanthanum carbonate is used as an efficacious Pi-binder in dialysis patients, even if it is minimally absorbed. On the other hand, intravenous gadolinium is used with magnetic resonance, but caution should be observed for patients with declined renal function.

About calcimimetics, several studies investigated the effects of these compounds in animal model with vascular calcification (154, 155), including cinecalcet, the only calcimimetic used in dialysis patients affected by secondary hyperparathyroidism, even if *in vitro* experimental

data are missing. The data of this study confirm that the activation of the CaSR at the vascular level may be important to prevent and reduce VSMC calcium deposition, by both an indirect effect on Ca^{++} and Pi levels and by a direct effect on CaSR activation (143, 144, 156, 157).

In summary, $LaCl_3$ prevents high Pi-induced vascular calcification *in vitro* by affecting VSMC osteoblastic differentiation. Several reports have shown that $LaCl_3$ prevents calcium deposition in VSMCs (158) and ameliorates vascular calcification in uremic rats (159, 160). In our study, we found that lanthanum (I) prevents high Pi-induced calcium deposition in VSMCs independently by Pi binding effect; (II) this reduction is a direct effect on VSMCs by preserving vascular phenotypic changes, preventing osteoblastic differentiation and affecting apoptosis.

In fact, lanthanum counteracts VSMC transdifferentiation, by preserving smooth muscle lineage markers and by delaying the transformation in osteoblastic-like cells through the inhibition of BMP-2 and osteonectin elevation. Probably, as a consequence, VSMCs don't need to activate some protective mechanisms against high-Pi, such as elevation in MGP and SPARC levels. Interestingly, $LaCl_3$ seems to act also in preventing apoptosis.

$GdCl_3$ does not share similar mechanisms of action of $LaCl_3$, except for BMP-2, and more studies are required to elucidate its action on vascular calcification.

The calcimimetic calindol prevents high Pi-induced VSMCs calcium deposition by affecting VSMC osteoblastic differentiation. In fact, calindol prevents vascular calcium deposition by delaying the transformation of VSMCs in osteoblastic-like cells through the inhibition of BMP-2 elevation. Nevertheless, VSMCs partially proceed in the differentiation process by loosing smooth muscle lineage markers and by increasing Cbfα1/RUNX-2 levels, in spite of calindol pretreatment. As a consequence, VSMCs need to activate some protective mechanisms against high Pi, such as elevation in MGP levels. Interestingly, calindol does not seem to act also in preventing apoptosis.

So, these three pharmacological treatments have the same final effect on VSMC calcification, because they are able to reduce calcium deposition in VSMCs challenged with high Pi, but their intracellular action is not the same because they modulate different proteins involved in different pathways all implicated in the pathological mechanism of vascular calcification.

8. CONCLUSIONS

First of all, we studied the modulation of a major non collagenous protein of bone matrix, osteonectin, in VSMCs challenged with high Pi for 7 days *in vitro* and in calcified arteries *ex-vivo*, finding results that suggest a pro-calcifying role of this protein. Indeed, our *in vitro* studies demonstrated that there is an association between osteonectin expression and high phosphate-induced vascular calcification, suggesting that this protein, probably, acts in VSMCs as a pro-calcifying agent and contributes to the process of osteoblastic differentiation. Furthermore, osteonectin expression in human arteries *ex-vivo* is increased in VSMCs at the site of arterial calcification.

Then, we investigated some of the potential biologic processes modulated by high Pi treatment that lead to the final event of VSMC calcium deposition, and we discovered that extracellular VSMC calcium deposition is the result of a multifactorial process that involves all a series of molecular and cellular changes, that culminate in VSMCs loss of their contractile phenotype in favour of a simil-osteoblastic transformation, in an increment of VSMC apoptosis and in inability of VSMCs to put to use protective pro-survival strategies, like autophagy, to try to save themselves from cell death.

Since high phosphorous has a relevant effect on VSMC calcification, and since cardiovascular disease is the most common cause of death in CKD patients, we investigated potential strategies to delay the progression of high Pi-induced VSMC calcium deposition.

First of all, we experimented the “Wash Out” treatment in our *in vitro* model of vascular calcification: surprisingly, we discovered that it is sufficient the total absence or the partial decrease of Pi concentration under a so called “trigger threshold” for short and repeated periods during the process of calcification to obtain a substantial inhibition of calcium deposition. We demonstrated that these protective effects are the result of slowing down molecular and cellular processes that are “switch on” by high Pi treatment: indeed, we can hypothesize that in the “Wash Out” period, when Pi levels are under “threshold”, cells freeze the biological processes that finally lead to calcium deposition because, temporarily, the action of Pi is “off”, and so they arrest osteoblastic transformation, apoptosis and “switch on” the autophagy protective mechanism. To obtain these benefits is not important the type of phosphorous they are challenged with (free Pi or hydroxyapatite) : what is important is that they are exposed to under threshold conditions.

Then, we investigated the mechanism of action of two drugs CKD patients are treated with in the attempt to reduce hyperphosphatemia and to contrast secondary hyperparathyroidism, respectively: Lanthanum Chloride ($LaCl_3$) and Calindol. We demonstrated that these drugs significantly delay the progression of high Pi-induced VSMC calcium deposition with different mechanisms of action: they shared only a mechanism to contrast vascular calcification, that is the delay of VSMC osteoblastic transformation; lanthanum is also able to partially prevent high Pi-induced apoptosis, while calindol "switch on" the positive modulation of a VSMC anti-calcific protein.

These *in vitro* discoveries can suggest that it is of relevant importance to control as more as possible phosphatemia in CKD patients, even with diet, because high Pi is the most dangerous key regulator of vascular calcification in end stage renal disease pathology: so, it should be opportune to increment the duration of haemodialysis cycles in CKD patients, coupling with, if it is necessary, an adequate therapy, to consent VSMCs to be in contact, as more as possible, with under threshold Pi levels.

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