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Ectopic calcification: from pathogenetic pathways towards therapeutic perspectives

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ABBREVIATIONS

3D: three-dimensional
ABCC6/Abcc6: ATP – binding cassette sub-family C member 6 (human/mouse)
ACDC: arterial calcification due to CD73 deficiency
ACP: amorphous calcium phosphate
aHDF: adult human dermal fibroblast
Ahsg: 2-Schmid Heremans glycoprotein
AMP: adesosine monophosphate
ANKH: ankylosis protein homolog
AST: alendronate sodium trihydrate
ATP: adenosine triphosphate
BBB: blood-brain barrier
BCP: biphasic calcium
BMP: bone morphogenetic protein
BSP: bone sialoprotein
Ca: calcium
CEC: circulating endothelial cells
CKD: chronic kidney desease
CPD: cumulative population doublings
CVD: cardiovascular disease
ECM: extracellular matrix
ENPP1/PC1: ectonucleotide pyrophosphatase/phosphodiesterase 1
EPC: endothelial progenitor cells
ETD: etidronate disodium
FBLN5: fibulin-5
FBS: foetal bovine serum
GACI: generalized arterial calcification of infancy
GH: growth hormone
Glu- and Gla-MGP: uncarboxylated- and carboxylated-matrix Gla protein
HA: hydroxyapatite
hBM-MSC: human bone marrow mesenchymal stem cells
HFTC: hyperphosphatemic familial tumoral calcinosis
hPL: human platelet lysate

IBGC: Idiopathic basal ganglia calcification

IGF-1: insulin-like growth factor 1

KO: knock-out

MGP: matrix gla-protein

mtDNA: mitochondrial DNA

MVs: matrix vescicles

NFTC: normophosphatemic familial tumoral calcinosis

nHDF: neonatal human dermal fibroblast

OC: osteocalcin

ON: osteonectin

OPG: osteoprotegerin

OPN/SPP1: osteopontin

PDGFB: platelet-derived growth factor beta

PDGFRB: platelet-derived growth factor receptor beta

Pi: inorganic phosphate

Pit-1,2: phosphate transporter-1,2

PPi: inorganic pyrophosphate

PXE: pseudoxanthoma elasticum

RANKL: receptor activator of nuclear factor kappa-B ligand

ROS: reactive oxygen species

SDF-1: stromal-derived factor-1

TNAP, tissue non-specific alkaline phosphatase

VC: vascular calcification

VEGF: vascular endothelial growth factor

VEGFR-KDR: endothelial growth factor receptor-2

VKCFD: vitamin K-dependent factors

VSMC: vascular smooth musle cells

WT: wild-type

ABSTRACT

Pathologic calcification of soft connective tissues is a frequent complication of age-related disorders (i.e. atherosclerosis and kidney diseases). Recent studies have demonstrated that mineral precipitates are the result of a dynamic process due to an altered production of pro- and anti-calcifying factors by mesenchymal cells. Due to the complex interactions between cells, structural molecules, soluble factors and genes, pathomechanisms of ectopic calcification are still elusive and no specific therapies are available. Within this context, inherited diseases such as Pseudoxanthoma elasticum (PXE), due to ABCC6 gene mutations, represent interesting models to investigate specific molecular pathways.

Aim of the present PhD dissertation was to better understand the role of mesenchymal cells in ectopic calcification.

Firstly, dermal fibroblasts from PXE and healthy subjects were cultured *in vitro* in the presence of pro-calcifying stimuli. Under these environmental conditions, fibroblasts alter their phenotype changing ENPP1/PC1 and SPP1/OPN expression, as well as TNAP activity, thus favouring matrix mineralization. Interestingly, in PXE cells, the ratio between factors favouring and reducing PPi availability (i.e. one of the most powerful inhibitor of the mineralization process) exhibits a more pronounced shift towards a pro-calcifying balance.

A still unsolved question is whether changes in fibroblasts' behaviour are the cause and/or the consequences of the calcification process. Investigation on dermal fibroblasts from Abcc6 transgenic mice of different ages indicated that these cells exhibit modifications related to the genotype, being detected well before the development of calcification (Ank and Opn down-regulation), whereas other changes (O_2^- content, Tnap activity, and Bmp2 up-regulation) may represent a cellular response to the calcified environment and/or the consequence of the ageing process *per se*.

To understand if cells are more responsive to ectopic calcification with ageing, thus contributing to the incidence of aberrant mineralization in the ageing population, fibroblasts from neonatal (nHDF) and adult (aHDF) subjects (*ex vivo* aging model) were cultured up to replicative senescence (*in vitro* aging model) in standard and in calcifying media. Results demonstrated that donor's age and replicative senescence play in concert increasing the calcification process and altering the ANKH+ENPP1/TNAP ratio, thus affecting PPi availability.

It could be suggested that age-related morpho-funcional alterations of soft connective tissues, including ectopic calcification, may be an additional consequence of "inflammaging", where soluble factors as well as circulating cells play a role. Studies in which human platelet lysate was added to cultured nHDF, aHDF and hBM-MSC indicate that platelet-released factors promote

mineralization, although with differences due to individual variability/susceptibility and/or to the methods used to prepare the lysate. In addition, in patients affected by vascular calcification, circulating endothelial and progenitor cells (i.e. cells involved in vascular damage and repair, respectively), being detected by high performance flow cytometer, exhibit a shift toward an osteoblast-like phenotype, thus paving the way for the clinical application of these cells as markers of vascular calcification.

Finally, in order to provide a 3D substrate for the growth of cells in the presence of small dispersed mineral precipitates, an enzymatically calcified collagen gel has been developed and characterized by SEM-FEG, whereas cell viability and morphology were evaluated by fluorescence microscopy. Further studies are in progress to evaluate the response of mesenchymal cells in these culture conditions.

1. Physiological mineralization

Physiological mineralization takes place during the formation and the development of mineralized tissues, e.g., bones and teeth (Thouverey et al., 2007; Kirsch et al., 2006). The vertebrate skeleton produces five different mineralized tissues: dental enamel, dentin, cementum, calcified cartilage, and bone. While the mineral content in each of these tissues varies, the mineralized phase is the same, consisting of a carbonate-rich hydroxyapatite, or containing small amounts of Na^{+}, K^{+}, Mg^{+2}, and Cl^{-}, termed biological apatite (Termine, 1972; Trautz, 1955). The organic phase of these tissues is composed primarily of collagens, except in enamel. Of the collagenous mineralized tissues, three are composed of mainly type I collagen, the exception being calcified cartilage which is built on a type II collagen matrix. All of the collagenous mineralized tissue matrices also contain non-collagenous proteins. The overall mechanism of mineralization of the four collagenous tissues is similar: cells secrete an unmineralized organic matrix and the mineral phase then accumulates in the space between the cells and the mineralization front. In addition to synthesizing and secreting the organic matrix, cells maintain the ion composition of the extracellular space through the action of Ca^{2+} and PO_4^{3-} ion pumps (Sosnoski et al., 2008; Palmer et al., 2001). The activity of these ions in the extracellular space is modulated by non-collagenous matrix proteins and by the presence of inorganic pyrophosphate (POP). The latter compound, at low concentrations, is a potent inhibitor of mineral formation. Non-collagenous proteins have been shown to enhance or to inhibit mineral formation, or do both (Boskey et al., 1989; Young et al., 1992). Thus, in the pre-mineralized matrix, conditions are poised for mineral to form, but the process is blocked by inhibitors. It is currently believed that mineralization is triggered by alteration of the PO_4^{3-}/POP ratio (Hessle et al., 2002). In addition, between intracellular and extracellular environment there is a careful regulation of Ca^{2+} and Pi. The cytoplasmic calcium concentration is approximately 0.1 μM. Cells pump Ca^{2+} out into the extracellular space. The ionic concentration of calcium in the extracellular space is modulated by the presence of non-collagenous matrix proteins (Talmage et al., 2007; Talmage et al., 2006). In contrast, cellular PO_4^{3-} levels are in the range of 5 mM as Pi is required for many metabolic reactions. Keeping the cytoplasmic Ca^{2+} concentration low allows cytoplasmic PO_4^{3-} concentration to be high without causing mineralization. Some of the POPs, however, are transported into the matrix via progressive ankylosis protein (ANK) (Gurley et al., 2006a; Kim et al., 2010).

The exact mechanism through which hydroxyapatite crystals form in vertebrate hard tissues has been widely debated (Glimcher et al., 1968; Wuthier et al., 1993).

The most likely mechanism proposed for bone and cartilage mineralization is based on the concept of heterogeneous nucleation (Glimcher, 1981). This mechanism relies on organic or inorganic precursor to direct the formation of apatite from soluble inorganic ions. Substantial differences exist

among authorities as to where this nucleation occurs and the exact molecular nature of the nucleator. Two models were proposed. One is the cell mediated model that proposes small vescicles, named matrix vescicles (MVs), at the site of initial or primary nucleation, as a prerequisite to subsequent secondary mineralization of the extracellular matrix (Figure 1) (Anderson, 1984; Boskey, 1992; Wuthier, 1989). These small particles (20–200 nm) are budded off from the plasma membrane of chondrocytes, osteoblasts, and odontoblasts prior to the onset of matrix mineralization. The composition of MV is different from that of the membranes from which they originate. The major components of MVs are reported in table 1.

Table 1. Major components of matrix vesicles (Golub et al., 2011).

Enzymes
Alkaline phosphatase (TNAP)
Phospho-1
Na+/K+ATPase
NPP1/PC-1
MMP-2
MMP-3
MMP-13
Transport proteins
Annexins 5, 2, 6, 11, 4, 1, 7
Pit 1,2
Other proteins
Integrins β1, β5, αV, α11, α1, α3
Lipids
Neutrals
Free fatty acids
Phosphatidylcholine
Phosphatidylethanolamine
Phosphatidylinositol
Phosphatidylserine

When levels of Ca^{2+} and PO_4^{3-} inside the vesicles become elevated, they begin to form $CaPO_4$ in non-crystalline form, called amorphous calcium phosphate (ACP; Wu et al., 1993). The ACP is converted into octacalcio phosphate, and then transformed into highly insoluble hydroxyapatite (Sauer and Wuthier, 1988).

The final stage of matrix mineralization is the deposition of very small crystals in the collagen fibrils. How these nanocrystals exit from the vesicle and reach the collagen fibrils is a matter of some debate. Alternatives view questions it has been proposed the occurrence of direct nucleation

of apatite by matrix macromolecules principally collagen, possibly involving phosphoproteins, phospholipids, and proteolipids (Raggio et al., 1986; Boyan et al., 1984; He et al., 2005). Further, studies on the behaviour of phosphoproteins *in vitro* are consistent with their role as nucleators or facilitators of nucleation (He et al., 2005; He et al., 2004). While each of these hypotheses for the initiation of mineralization is plausible and backed by a substantial body of evidence, no one has been able to fully explain all of the known features of mineralized tissue calcification.

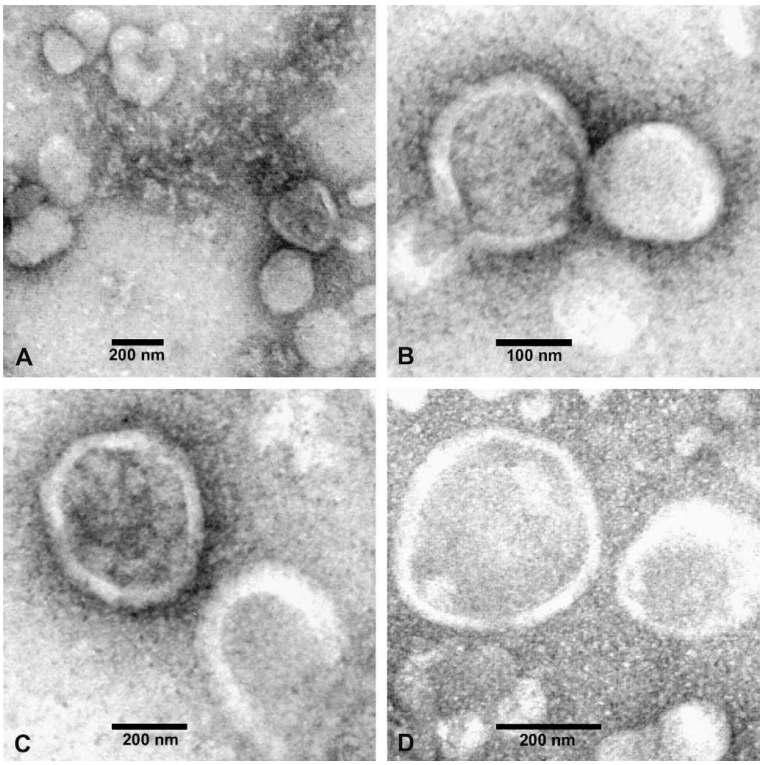

Fig. 1.**Trasmission electron microscopy of MVs.** MVs exhibit spherical shapes with a 100-250 nm diameter (Thouverey et al., 2009).

2. Ectopic calcification

Ectopic mineralization is a complex process leading to deposition of calcium-phosphate complexes in soft connective tissue. Particularly affects the skin and the arterial blood vessels and is common in aging and in age-associated disorders (Li et al., 2013) such as atherosclerosis, diabetes and renal disorders, contributing to increased cardiovascular morbidity and mortality (Murshed et al., 2010). A number of initiating and contributing metabolic and environmental factors are linked to aberrant mineralization, making the identification of precise pathomechanistic pathways exceedingly difficult. Many recent studies pointed out that calcium and phosphate precipitation are the results of complex and highly regulated series of events in which the balance between pro- and anti-calcifying factors may become severely deranged locally and/or systemically (Giachelli, 1999). The deposition of calcium and phosphate in soft connective tissues can be classified into three major categories:

metastatic calcification, dystrophic calcification, and calcinosis (Black and Kanat, 1985). Metastatic calcification occurs when calcium-phosphate levels are elevated mainly due to metabolic/hormonal alterations and/or to tumor-associated complications. Dystrophic calcification takes place in the presence of damaged or necrotic tissue as in atherosclerosis. Calcinosis is generally associated to hypovascularity or hypoxia, it may involve a localized area or it may be widespread, causing secondary muscle atrophy, joint contractures and skin ulceration, with recurrent episodes of inflammation or infection (Boulman et al., 2005).

Ectopic mineralization represents the consequence of several contributing metabolic and environmental factors, making the uncovering of the precise basis of these disorders in the populations at large exceedingly difficult (Li et al., 2013).

Several Mendelian genetic disorders share phenotypic similarities with the acquired forms of metastatic and dystrophic calcification, and serve as genetically controlled model systems to study various facets of pathological mineralization. Recent studies on these conditions, with accompanying animal models, allowed the identification of several genetic factors contributing to ectopic mineralization and provide evidence of intricate mineralization/anti mineralization networks in peripheral connective tissues. Moreover, recent studies demonstrated that mesenchymal cells are key players in ectopic calcification not only because they synthesize most of the mineral regulatory proteins, but also because they are responsible for the qualitative and quantitative characteristics of the extracellular environment, where apatite ectopic deposition arises (Ronchetti et al., 2013). In this contest very important is the extracellular matrix (ECM), changes in the characteristics of ECM (for example with aging) and the ratio between matrix constituents influence not only the mechanical properties of connective tissues, but also contribute to modulate cell phenotype by altering integrin expression, focal adhesions, cytoskeletal organization and consequently intracellular signaling pathways. Despite the apparent heterogeneity of factors contributing to the development of ectopic calcification, it is believed that there is a common pathogenetic mechanism.

The observation that MV-like membranes are present in a number of ectopic calcification processes supports the concept that the mechanisms of ectopic calcification are similar to those seen in normal skeletal development (Golub, 2011). Mineralization of dermal constituents, for instance, has been never associated with MVs, indicating that fibroblasts, differently from smooth muscle cells, can be responsible for mineral deposition, even in the absence of MVs. It could be, therefore, hypothesized that the role of mesenchymal cells in ectopic calcification may differ, depending on the ability of the cell type to acquire a bone-oriented phenotype (Ronchetti et al., 2013).

2.1 Factors involved in pathological calcification

On the contrary of physiological condition in which there is a fine regulation between inhibitor and promoter factors of calcification, in the pathological calcification this balance is less, and the deposition of minerals occurs.

In recent years, numerous works show that there are many molecules that regulate the homeostasis of calcium and phosphate. Initially, the molecules studied were those that regulate the homeostasis of calcium (catecholamines, parathyroid hormone and vitamin D) (Rizzoli et al., 1998). However, many other molecules can come into play during a calcification process and can be secreted by mesenchymal cells or spread through the bloodstream to peripheral tissues (Figure 2).

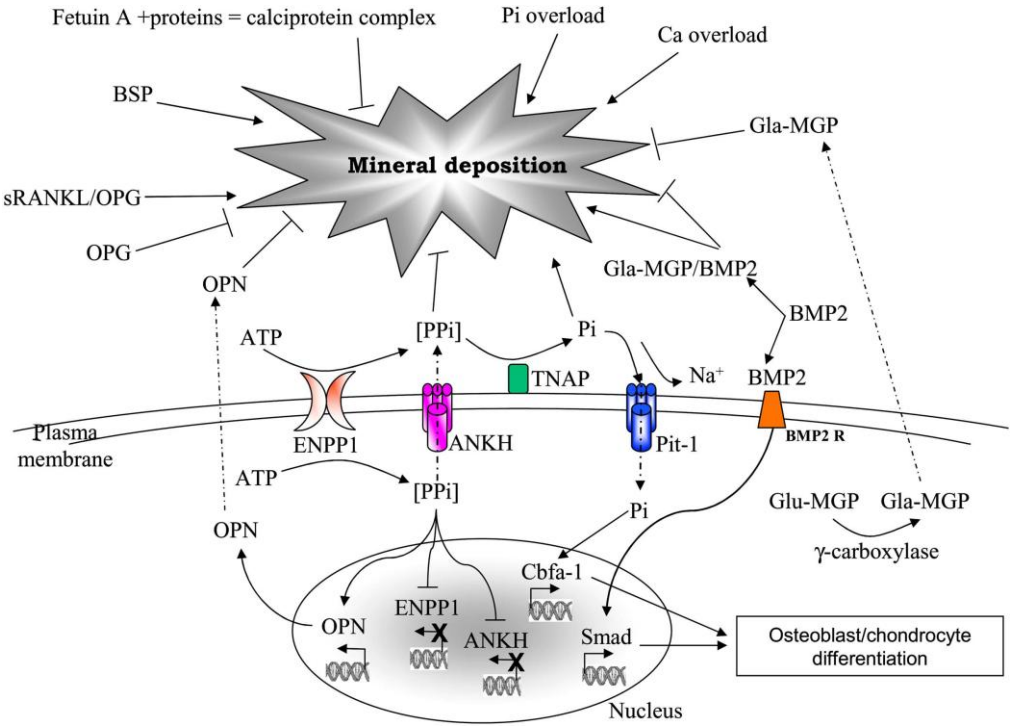

Fig. 2. Major factors involved in mineral deposition. AMP, adesosine monophosphate; ANKH, ankylosis protein homolog; ATP, adenosine triphosphate; BMP2, bone morphogenetic protein-2; BMP2R, bone morphogenetic protein-2 receptor; BSP, bone sialoprotein; Ca, calcium; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase; Glu- and Gla-MGP, uncarboxylated- and carboxylated-matrix Gla protein; OPG, osteoprotegerin; OPN, osteopontin; Pi, inorganic phosphate; Pit-1, phosphate transporter-1; PPi, pyrophosphate; RANKL, receptor activator of nuclear factor kappa-B ligand;TNAP, tissue non-specific alkaline phosphatase (Ronchetti et al., 2013).

- *Ectonucleotide pyrophosphatase/phosphodiesterase 1* (ENPP1): It is a trans-membrane glycoprotein composed of two identical subunits linked by disulfide bridges. ENPP1 is highly expressed in the bone, cartilage and teeth and appears to be the major enzyme responsible for the generation of extracellular/intracellular PPi from nucleoside triphosphate such as adenosine triphosphate (ATP) (Villa-Bellosta and Sorribas, 2013). Inactivating mutations of ENPP1 are associated with generalized arterial calcification of infancy, a

spontaneous form and often lethal calcification of the arterial tunica media (Rutsch et al., 2003). This suggests that vascular calcification can be also a spontaneous process regulated by inhibitors of calcification. The expression of ENPP1 is therefore essential to maintain the vascular integrity (Michel et al., 2011). ENPP1 common variants, as well as polymorphism K121Q, are associated with childhood obesity (Meyre et al., 2005; Bottcher et al., 2006), increased risk of type 2 diabetes (Meyre et al., 2005; Abate et al., 2005; Bacci et al., 2005; Bochenski et al., 2006; Willer et al., 2007), early development of diabetic nephropathy (Hanani et al., 2002) and myocardial infarction (Bacci et al., 2005; Endler et al., 2002). Also, it was seen that patients with GACI bearing mutations of the ENPP1 gene, may develop typical signs of PXE; therefore, mutations on ABCC6 and ENPP1 seem to determine alterations in the same molecular pathways (Nitschke et al., 2012; Li et al., 2012).

- *2-Schmid Heremans glycoprotein (Ahsg) or Fetuin-A:* it is a member of the cystatin superfamily, inhibitors of cysteine proteases (Elzanowski et al., 1988). Identified for the first time in fetal bovine serum (FBS). It is a glycoprotein produced by the liver acting as an inhibitor of systemic mineralization. Fetuin-A is produced in the adult liver and is secreted in the circulation, but it is also expressed by many other organs during embryogenesis (Terkelsen et al., 1998). In addition, to being an important carrier protein complex of calcium-phosphate in the blood, where it prevents the precipitation (Quelch et al., 1984), it is also transported into the bone where it is one of the most abundant non-collagenous protein (Reynolds et al., 2005). Fetuin accumulates in the skeleton because has an high affinity for hydroxyapatite (Triffitt et al., 1978; Schinke et al., 1996). Some studies suggest that Ahsg inhibits only transiently (for several hours) *de novo* formation and precipitation of mineral apatite precursors (essentially biphasic calcium (BCP)), but it is not capable of dissolving the BCP once it is formed (Schinke et al., 1996; Heiss et al., 2003). The inhibitory effect on calcification was demonstrated in knock-out mice, which suffer from severe extra-osseous mineralization (Schaefer et al., 2003). The fetuin-A inhibits calcification by binding to the particles of calcium-phosphate that have just been formed, facilitating the elimination and thereby impeding crystals growth and minerals deposition. Although it has been widely shown that low serum fetuin-A is associated with the presence of vascular calcifications and increased mortality in people with kidney disease, further studies are needed to exactly understand the role of fetuin-A in vascular calcifications (Tousoulis et al., 2012).
- *Alkaline phosphatase tissue-non specific* (TNAP): it is a cell membrane-bound ectoenzyme that increases inorganic phosphate availability by releasing it from a variety of phosphate-

enriched substrates and, at the same time, reduces the levels of calcification inhibitors, promoting the hydrolysis of PPi and the dephosphorylation of osteopontin (OPN; El-Abbadi et al., 2009; Orimo, 2010). Consistently, increased TNAP expression and activity have been observed in CD73 deficiency, a disorder that, due to mutations in NT5E, is characterized by tortuosity and calcification of lower limb arteries and by mineralization of hand and foot joint capsules (St Hilaire et al., 2011). In the vascular system TNAP, together with the ENPP1, controls calcification by acting on the synthesis and hydrolysis of PPi (Villa-Bellosta et al., 2011). The role of TNAP in the calcification process has been demonstrated in patients with hypophosphatasia, an inherited disease, linked to the X chromosome that causes a deficiency of TNAP and leads to defective bone formation (Whyte, 1994; Stonich et al., 2009). A similar condition can also occur as a result of mutations involving a calcium binding site with an abnormal protein folding (Mornet et al., 2001).

The expression of this enzyme is clearly associated to the calcifications, either physiological or pathological, even if one of its different activities is not directly and necessarily accompanied by changes in levels of mineralization, which are in fact the result of the combined action of several genes / proteins (Mendes et al., 2004).

- *Bone morphogenetic protein 2* (BMP2): it is a powerful cytochine that, by activating Smad signaling pathways, promotes *in vitro* differentiation of mesenchymal cells into osteoblasts and induces *in vivo* bone formation (Rosen, 2009). In particular BMP-2, -6, -7 and -9 are considered multifunctional cytokines in bone remodelling and osteogenesis, favouring bone differentiation (Luu et al., 2007). During osteogenic process, BMPs bind to heterodimeric transmembrane receptors (BMP receptor type IA, IB and type II) (Singhatanadgit et al., 2006) causing the activation of signalling molecules in different pathways (Chen et al., 2004; Sapkota et al., 2007; Chan et al., 2007; Ikeda et al., 2007; Lian et al., 2006; Wang et al., 2007; Chen et al., 2007).

  According to some studies, BMP7 slows the process of calcification, while BMP4 and BMP2 would be involved in the induction of local inflammation and mineralization in the vascular tissue. In addition, the activity of BMP2 is regulated by Matrix Gla Protein (MGP) in dose dependent way. Therefore according to ratio of the MGP and BMP2 it is possible to induce or to inhibit the calcification process (Zebboudj et al., 2002).
- *Progressive ankylosis protein homolog* (ANKH): it is a transmembrane protein of 492 amino acids, which carries inorganic pyrophosphate through the plasma membrane into the extracellular environment (Ho et al., 2000; Nürnberg et al., 2001; Terkeltaub, 2001). PPi regulates cellular differentiation, acting as a physiological inhibitor of calcification and its

presence in the extracellular level is the result of ANKH activity. It is under the control of TGF-β1 and is influenced by extracellular calcium concentration (Cailotto et al., 2007). The function of ANK is therefore essential, especially in the junctions where it helps to prevent the formation of hydroxyapatite crystals, preserving joint mobility (Gurley et al., 2006b; Masuda and Hirose, 2002).

ANKH loss of function, due to spontaneous mutations, leads to decreased PPi extracellular levels (Ho et al., 2000; Harmey et al., 2004; Johnson et al., 2003; Gurley et al., 2006b), as an increase of mineral formation in the articular cartilage it has been observed in a disease called progressive ankylosis. In contrast, ANKH overexpression, *in vitro*, leads to increased extracellular pyrophosphate (Ho et al., 2000; Zhang et al., 2005), thus inhibiting the calcification process.

- *PPi*: it is a small molecule made of two phosphate ions, linked by an ester bond, that regulates cell differentiation and serves as an essential physiologic inhibitor of calcification by negatively interfering with crystal growth (Terkeltaub, 2001). The amount of extracellular PPi is regulated by two different gene products, as it originates either from the breakdown of nucleotide triphosphates by the ectonucleotide pyrophosphatase phosphodiesterase 1 or from the PPi transport by the transmembrane ankylosis protein homolog.
- *Osteonectin* (ON): it is a secreted, Ca^{2+} ion-binding, glycoprotein that is found in a variety of different cell types including osteoblast, endothelial cells, ligament fibroblasts, parietal yolk sac cells and in basement membrane tumors. As its name suggests, osteonectin was originally identified as a constituent of bone extracellular matrix (Termine et al., 1981). The reported ability of bone-derived osteonectin to bind Ca^{2+} and type I collagen and to inhibit hydroxyapatite crystal growth is suggestive for a major role in bone mineralization (Romberg et al., 1986; Doi et al., 1989).
- *Osteopontin* (OPN): it is an acid phosphorylated glycoprotein (Senger et al., 1989). OPN is a multifunctional protein which contains several structural domains, as those responsible for integrin binding as well as regions rich in aspartic acid. The combination of electronegative residues of glutamic acid and aspartic acid, which may act as a substrate for serine / threonine kinase and which are the presumed binding domain for calcium, provides to OPN the ability to bind significant amounts of Ca^{2+} (Chen et al., 1992). Post-translational modifications, by phosphorylation of serine and threonine, and O-glycosylation, can lead to OPN various functional variants (Singh et al., 1990; Christensen et al., 2007; Christensen et al., 2005), but it should be noted that the protein can also be functionally modulated by

proteolytic cleavage. It is present in many tissues, body fluids (Yoon et al., 1987; Swanson et al., 1989; Waterhouse et al., 1992; Senger et al., 1989; Sørensen et al., 2003; Franzen and Heinegard , 1985; Denhardt et al., 2001; Renkl et al., 2004) and in mineralized tissues (such as bone and teeth) where it acts inhibiting hydroxyapatite crystal growth or promoting osteoclast activity (Giachelli et al., 2000). Osteopontin levels are increased in patients with severe kidney disease, with ectopic calcification and autoimmune diseases. It has been suggested that an increase in osteopontin plasma levels is associated with the presence of calcification in aortic valve stenosis, thus suggesting that it may play a functional role in the pathogenesis of this pathologic condition (Yu et al., 2009). Nevertheless, there are contradictory data on the role of the protein in atherosclerotic plaques and in mineralized aortic valves, and it has been hypothesized that OPN could be also involved in active regression of calcified areas (Ohri et al., 2005).

- *Osteocalcin* (OC): it is secreted into the bone micro-environment and then undergoes a conformational change that aligns calcium-binding Gla residues with the calcium ions in hydroxyapatite. This property was initially proposed as a mechanism enabling osteocalcin to initiate the formation of hydroxyapatite crystals (Price et al., 1976). However, subsequent work was more compatible with the notion that osteocalcin functions as an inhibitor of bone mineralization. In support of this idea, osteocalcin inhibits the precipitation of calcium salts from saturated solutions (van de Loo et al., 1987).
- *Osteoprotegerin* (OPG): it is a key regulator in bone metabolism, but it has also an effect on the vascular system. Studies suggest that osteoprotegerin is a critical arterial calcification inhibitor, and is released by endothelial cells as a protective mechanism for their survival in certain pathological conditions, such as diabetes mellitus, chronic kidney disease, and other metabolic disorders. It is produced by bone marrow mesenchymal cells of and plays a central role in regulating bone turnover by inhibiting osteoclast differentiation (Boyle et al., 2003; Van Campenhout et al., 2009). It may act, as a receptor for the ligand NF-kB (system RANKL) (Lacey et al., 1998) interfering with the interactions between RANK (expressed by osteoclast-like cells) and RANKL (expressed by osteoblast-like cells). OPG inhibits the activity of alkaline phosphatase (Bennett et al., 2006), reduces the mobilization of calcium and phosphate and plays a protective role against ectopic calcification (Van Campenhout et al., 2009).
- *Matrix Gla-Protein* (MGP): it is a single polypeptide chain of 10 kDa molecular weight encoded by the MGP gene. It is composed of 79 amino acid residues of which nine are of glutamic acid; of these, five are post-translationally modified by a process known as γ-

carboxylation, which occurs in the endoplasmic reticulum, being cathalized by the presence of vitamin K (Price et al., 1985). It was observed that, both *in vivo* and *in vitro*, MGP is constitutively expressed in many cell types, such as fibroblasts and smooth muscle cells where it acts as an inhibitor of local ectopic calcification, in cartilage and vascular tissue (Ketteler et al., 2005). Where MGP is synthesized as a non-carboxylated form and, if there is poor vitamin K availability, it remains non-carboxylated and loses its ability to inhibit calcification. The carboxylation degree necessary to inhibit calcification in humans is still unknown (Schurgers et al., 2007; Fu et al., 2008). It was demonstrated that MGP prevents arterial calcification by directly interacting with crystals of HA (O'Young et al., 2011), and/or by acts indirectly influencing transcription factors able to counteract the differentiation of cells (*e.g.*VSMC) towards an osteoblast-like phenotype (Bostrom et al., 2001). Individuals suffering from Keutel syndrome have mutations in the MGP gene and develop calcifications in the tunica media of blood vessels and in cartilage (Meier et al., 2001; Munroe et al., 1999; O'Young et al., 2011). Moreover, it has been observed that MGP polymorphisms are associated with coronary artery calcification in men (Crosier et al., 2009).

- *Bone Sialoprotein* (BSP) is a non-collagenous protein undergoing several post-translational modifications (phosphorylation, glycosylation and sulfation). It is localized on collagen fibrils, where acts as a nucleation site for hydroxyapatite crystals. BSP is transcriptionally regulated by hormones, growth factors and cytokines through tyrosine kinase and cAMP-dependent pathways. Being important in osteoblast differentiation, in bone matrix mineralization and in tumor metastasis (Ogata, 2008).
- *Phosphate transporter 1* (Pit-1) is located on the cell membrane and on the endoplasmic reticulum (ER) (Villa-Bellosta et al., 2011). It is part of the phosphate sodium-dependent co-transporters regulating phosphate entrance within the cells. Several receptors have been identified as type I, II and III and Pit-1 that are widely expressed in different cell types including osteoblasts, chondrocytes, and smooth muscle cells (Jono et al., 2000; Boyer et al., 1998 ; Kakita et al., 2004; Palmer et al., 1997; Kavanaugh et al., 1994; Li et al., 2006). This protein, by activating the Erk1/2 signaling pathway promotes calcification and differentiation of VSMC to an osteo-chondrogenic phenotype. Studies conducted on VSMC revealed that, when cells are treated with PDGF, thus increases the expression of Pit-1 on ER. Therefore, Pit-1 could alter not only the Pi flow within the cell, but it may also modify the expression of ER proteins such as MGP or kinases involved in the protein phosphorylation as in the case of OPN (Villa-Bellosta et al., 2011).

2. 2 Mesenchymal cells in soft connective tissue mineralization

Changes in the characteristics of the extracellular matrix and in the ratio between matrix constituents influence not only the mechanical properties of connective tissues, but significantly contribute to modulate cell phenotype by altering integrin expression, focal adhesions, cytoskeletal organization and consequently intracellular signaling pathways. Therefore, beside alterations in the balance between pro- and anti-calcifying factors, changes in the extracellular matrix may significantly contribute to mineral deposition.

Recent studies demonstrated that mesenchymal cells are key players in the ectopic calcification not only because they synthesize most of the mineral regulatory proteins, but also because they are responsible for the qualitative and quantitative characteristics of the extracellular environment, where apatite ectopic deposition arises (Ronchetti et al., 2013).

In the last years many reserchers have focused their attention on the role of mesenchymal cells in the ectopic calcification and today we can affirm that these cells are protagonists in ectopic calcification.

Pericites

Some studies proposed that they may derive from the neural crest, or from smooth muscle cells, fibroblasts, endothelial and bone marrow cells and that they exhibit a multi-lineage potential, being capable of differentiating into a variety of cell types including osteoblasts and chondrocytes, as demonstrated, both *in vitro* and *in vivo* experimental models. On the basis of these observations, it was suggested that pericytes play a role in mediating ectopic calcification (Collett and Canfield, 2005).

The association between angiogenesis and ectopic calcification has been noted in a number of carcinoma *in situ*, in calcifying fibroblastic granuloma, in choroidal osteoma and in the calcifications of the retina. When cultured in standard growth medium, pericytes undergo a process of growth and differentiation characterized by the formation, within approximately 8 weeks, of large multi-cellular nodules that, similarly to the matrix found in calcified vessels, contain type I collagen, OPN, matrix Gla protein and OC and hydroxyapatite crystals with a calcium to phosphate ratio analogous to that of bone (Doherty and Canfield, 1999; Abedin et al., 2004).

Vascular smooth muscle cells

It has been already demonstrated that the cardiovascular system undergoes progressive morpho-functional changes with age, as fragmentation of elastic laminae, increased collagen deposition and altered cell-matrix alterations contributing to modify elasticity and resilience of valves and blood vessels. Since the pro-osteogenic phenotype of vascular smooth muscle cells (VSMCs) is associated to a shift from a contractive to an osteogenic signature, it could be suggested that changes in the

environmental milieu and/or the contractile potential of cells could influence the susceptibility of a tissue to favour/inhibit pathologic mineral deposition. Interestingly, it has been shown that, in appropriate culture conditions, approximately 10-30% of VSMCs have the capacity to express osteoblast differentiation markers and to retain this phenotype through several *in vitro* passages (Boström et al.,1993). In addition, *in vivo* and *in vitro* experiments demonstrate that also oxidative stress promotes the expression of gene related to osteogenic differentiation in VSMC (Parhami et al., 2002).

Recently, studying human coronary artery smooth muscle cells, we have demonstrated that differentiated contractile cells are more resistant to hydroxyapatite deposition and that, in the balance between pro- and anti-calcifying factors a threshold level of TNAP activity must be reached in order to promote ectopic calcification (unpublished data).

Fibroblasts

Many studies on VSMCs have shown that mesenchymal cells, being involved in extracellular matrix synthesis and degradation and producing pro- and anti-calcifyng factors are involved in connective tissue calcification.

On the contrary,only few studies have been performed on fibroblasts, and in particular on dermal fibroblasts, even if, fibroblasts are directly involved in pathologic calcification.

Fibroblasts are cells more represented in the connective tissue and are a very heterogenous population according to anatomical site (Shim et al., 2013).

It has been demonstrated that human gingival fibroblasts may exhibit both intracellular and extracellular mineral deposits, that start to form within round and irregularly shaped vesicles contained in large cytoplasmic vacuoles (Yajima et al., 1984). By contrast, MV have been never observed within or around dermal fibroblasts in areas of matrix calcification *in vivo* nor in the high phosphate-calcification model *in vitro*. This finding indicates the occurrence of different phenotypic characteristics between dermal and gingival fibroblasts, but, at the same time, it provides evidence that mineral deposition can be observed also independently from MV.

Yu and coworkers performed a cDNA microarray analysis on fibroblasts from spinal ligaments cultured in the presence of conditioned media from osteoclast-like cells. In this environment fibroblasts exhibited high levels of alkaline phosphatase and mineral deposition, but more interestingly, microRNA expression profiles revealed a significant down-regulation of a group of microRNAs known to negatively interfere with genes associated with osteogenic differentiation (e.g., BMP2, OC, Runx2).

In the light of these data, it has been hypothesized that osteoclasts might induce the osteogenic differentiation of fibroblasts *in vitro* and that miRNA may play an important role in the regulation of cell-cell interactions between osteoclasts and fibroblasts (Yu et al., 2011).

An additional demonstration of the ability of fibroblasts to modulate their phenotype in response to specific environmental characteristics has been provided by studies on rat dermal fibroblasts cultured in the presence of TGFβ-1 and of elastin degradation products. These fibroblasts produced a calcified matrix as consequence of increase of osteogenic markers such as osteocalcin, alkaline phosphatase and osteoprotegerin (Simonescu et al., 2007).

It is important to note that a specific environment is required for calcification to occur *in vivo*, but especially *in vitro*.

As already demonstrated, a standard cell culture environment is unsuitable to promote mineralization *in vitro*, independently from the ability of cells to induce calcification *in vivo* (Irie K, et al., 1998). To promote this process *in vitro* it is possible to add high phosphate concentrations (that can easily precipitate as soon as it forms complexes with calcium) or phosphate donor substrates (that require an active involvement of cells for the enzymatic release of inorganic phosphate from substrates). According to Buranasinsup (2006) human dermal fibroblasts can acquire an osteoblast-like behavior and can start to mineralize if cultured in a pro-osteogenic medium (supplemented with ascorbic acid, β-glycerophosphate and dexamethasone).

This type of medium is also able to counteract the inhibitory effects of proteins, such as fetuin A, present in serum supplements required for cell proliferation and survival. Fetuin A, represents a powerful inhibitor of the calcification process making cells unable to mineralize in standard cell culture conditions. In particular, dermal fibroblasts, cultured *in vitro* in a pro-calcifiyng envirnment produce a mineralized matrix after 2-3 weeks of culture (Boraldi et al., 2013). On the contrary other mesenchymal cells (*i.e*. osteoblasts and VSMCs) require fewer days to calcify (3-5 days or 8-10 days respectively; Uchimura et al., 2003; Li et al., 2004). These data indicate that fibroblasts are more reistent compared to other cells type to differenziate into osteoblast-like cells in agreement with the rare occurrence of calcifications in the dermis.

Adipocytes

Interestingly a mutual transdifferentiation between osteoblasts and adipocytes, has been observed both *in vitro* and *in vivo* (Nuttal et al., 1998), moreover adipose and bone tissues can influence themselves reciprocally. In particular, the first is considered an endocrine organ secreting adipokines (leptin, adiponectin) and hormones (estrogen, vitamin D3, androsterone, cortisone) some of which such, as leptin, are able to regulate bone metabolism by acting on cell transdifferentiation

(Savopouloset al., 2011). Alterations in the adipocyte differentiation and in their transdifferentiation in osteoblasts can cause ectopic calcification (Mohanty et al., 2010).

Condrocytes

In the litereture there are numerous articles demonstrating, both *in vitro* and *in vivo*, that condrocyte can differentiate into osteoblastic-like cells (Ishizeki et al., 1998; 2001; 2009), expressing elevated phosphatase alkaline activites as well as high osteocalcin, osteonestin, osteopontin and collagen of type I expression (Roach, 1992).

3. Ectopic calcification in genetic diseases

Significant advances in the understanding of the ectopic mineralization process have been made through the study of Mendelian single-gene disorders with phenotypic similarities with the "acquired" forms of metastatic and dystrophic calcification. These studies have allowed the identification of discrete pathways and factors involved in connective tissue mineralization. These studies have provided evidence for the presence of a complex mineralization/anti-mineralization network present in peripheral connective tissues (Rutsch et al., 2011; Li and Uitto, 2013) (Figure 3).

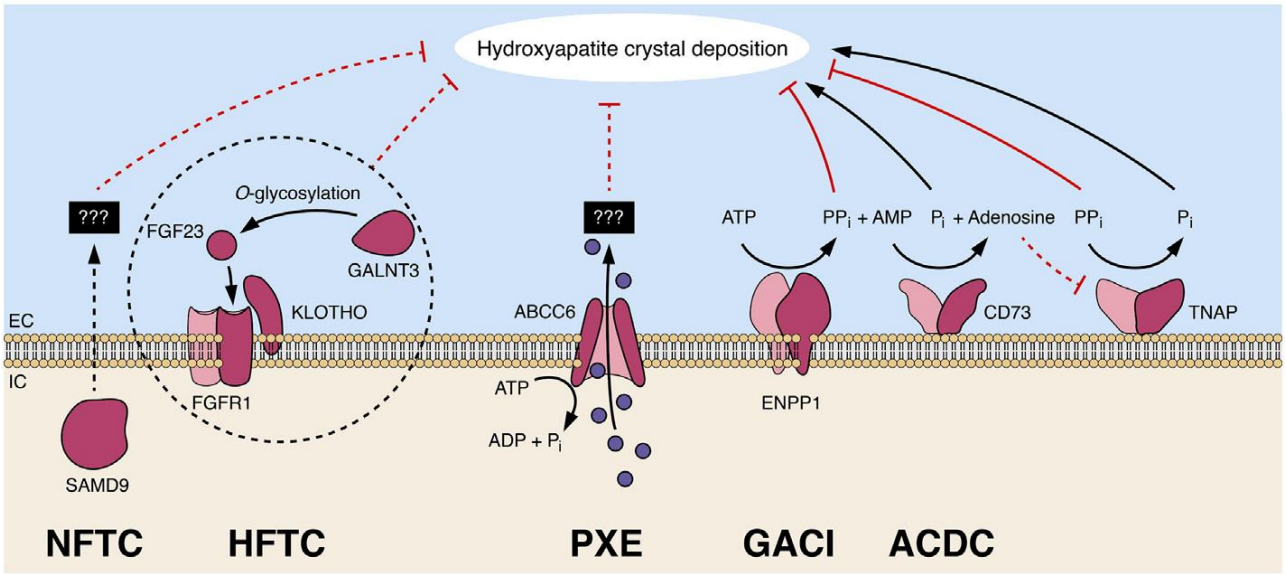

Fig. 3. Genetic complexity of the mineralization/anti-mineralization network in connective tissues. Mutations in specific genes can contribute to deposition of hydroxyapatite in heritable ectopic mineralization disorders: NFTC, normophosphatemic familial tumoral calcinosis; HFTC, hyperphosphatemic FTC; PXE, pseudoxanthoma elasticum; GACI, generalized arterial calcification of infancy; ACDC, arterial calcification due to CD73 deficiency. The blue solid circles represent currently unidentified anti-mineralization factors physiologically transported by ABCC6 from intracellular milieu to the extracellular space (Adapted from Li and Uitto, 2013).

Pseudoxanthoma elasticum

Pseudoxanthoma elasticum (PXE), a multisystem ectopic calcification disorder, is characterized by a progressive deposition of calcium-phosphate complexes in the extracellular matrix of soft connective tissues. Clinically, PXE presents alterations in skin, eyes, and the cardiovascular system (Neldner, 1988). Early skin manifestations consist of small yellowish papules, which progressively coalesce into larger plaques of inelastic and leathery skin. Histopathology of skin lesions reveals early accumulation of pleiomorphic elastic structures which progressively become mineralized.

Skin findings by themselves are primarily of cosmetic concern, but they signify the potential for development of serious eye problems and cardiovascular involvement (Georgalas et al., 2011; Leftheriotis et al., 2013). The characteristic eye lesions consist of angioid streaks which reflect the rupture of the mineralized Bruch's membrane, an elastin rich sheath between the pigmented retina and choroid. Angioid streaks are associated with broken eye capillaries resulting in bleeding, scarring, and neovascularization, causing progressive loss of visual acuity up to legal blindness. The cardiovascular system is also affected in PXE by progressive mineralization of medium sized arterial blood vessels, clinically resulting in hypertension, intermittent claudication, occasional bleeding from the intestinal arteries, and, rarely, in premature myocardial infarcts and stroke.

The inheritance of PXE is autosomal recessive with underlying mutations, in the ABCC6 gene encoding a putative transmembrane efflux transporter protein, MRP6 (Uitto et al., 2010). The ABCC6 gene is expressed primarily in the liver but is essentially undetectable in tissues directly affected by mineralization (Belinsky and Kruh, 1999). It has been postulated that MRP6, which is localized to the basolateral surface of hepatocytes (Pomozi et al., 2013), serves as a pump possibly involved in the transport of anti-mineralization factors from the liver into the circulation. In the absence of functional MRP6 activity, serum levels of such factors are reduced, allowing slow, yet progressive mineralization to ensue. It should be noted, however, that the precise nature of the molecules transported by MRP6 is currently unknown (Uitto et al., 2013). The estimated prevalence of PXE is ~1:25,000. Approximately 90% of PXE patients have mutations in the ABCC6 gene, and over 300 distinct loss-of-function mutations have been encountered; these include recurrent p.R1141* and p.A999_S1403del (del23-29) which account for ~40% of all mutations (Pfendner et al., 2007).

Murine model of PXE

The ability to reproduce human diseases in animals is a great advantage for the modern experimental medicine. The ideal animal model is the homologous that mimics the human disease in almost all features. Alternatively, the animal model can also be isomorphic, when reproducing

the disease, but not the underlying etiology, or predictive in the case where it is not particularly similar to the human disorder but allows to make predictions on it or on the response to treatment. Animal models have provided a great deal of knowledge, moreover they are a useful pre-clinical model for studying the pathogenesis and to test potential treatments.

As for PXE, the Abcc6$^{-/-}$ (KO) mouse was developed by targeted ablation of exons 15-18, through homologous recombination (Figure 4).

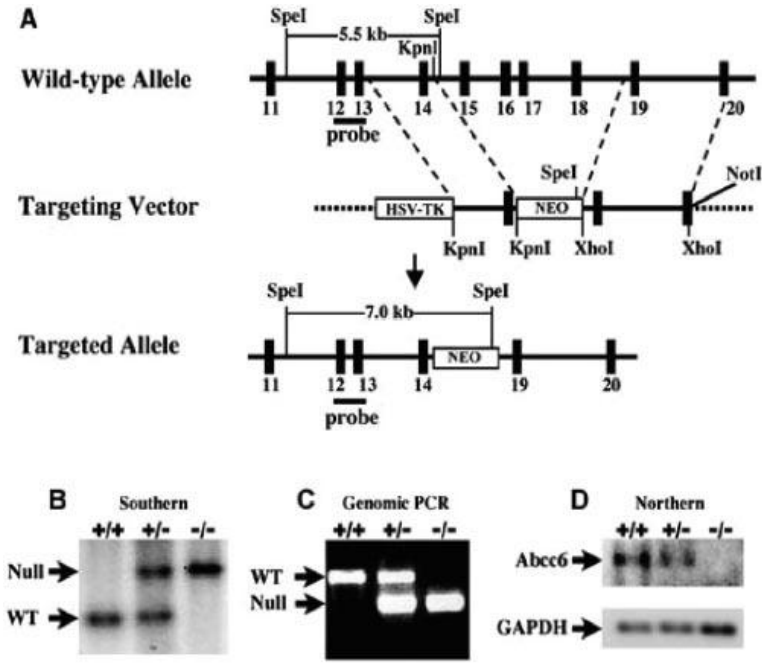

Fig. 4. Gene targeting in mice of Abcc6 locus. (A) Scheme of the murine Abcc6 gene (exons 11-20) and the targeting vector containing the HSV-TK gene and neomycin resistance (NEO) b) Southern blot analysis of DNA isolated from the tails; c) PCR amplification of genomic DNA; d) Northern blot analysis of a ~5 kb mRNA present only in the healthy mouse (+/+) and in the heterozygote (+/-) confirmed (below) for analysis with GADPH (glyceraldehyde-3-phosphate dehydrogenase) (Klement et al., 2005).

These mice recapitulate the genetic, histopathological and ultrastructural features of the disease. Consistently these mice develop progressive mineralization starting at ~5-6 weeks of age (Klement et al., 2005; Gorgels et al., 2005). The first site of mineralization is the connective tissue capsule surrounding the bulb of vibrissae in the muzzle skin, and this mineralization process has been shown to be progressive throughout mice life (Jiang et al., 2007). In addition to skin, mineralization has been shown to take place in the retina, arterial blood vessels, heart and kidneys (Klement et al., 2005). Although heart and kidneys of Abcc6$^{-/-}$ mice do not have extensive mineralized regions as the vibrissae (Figure 5).

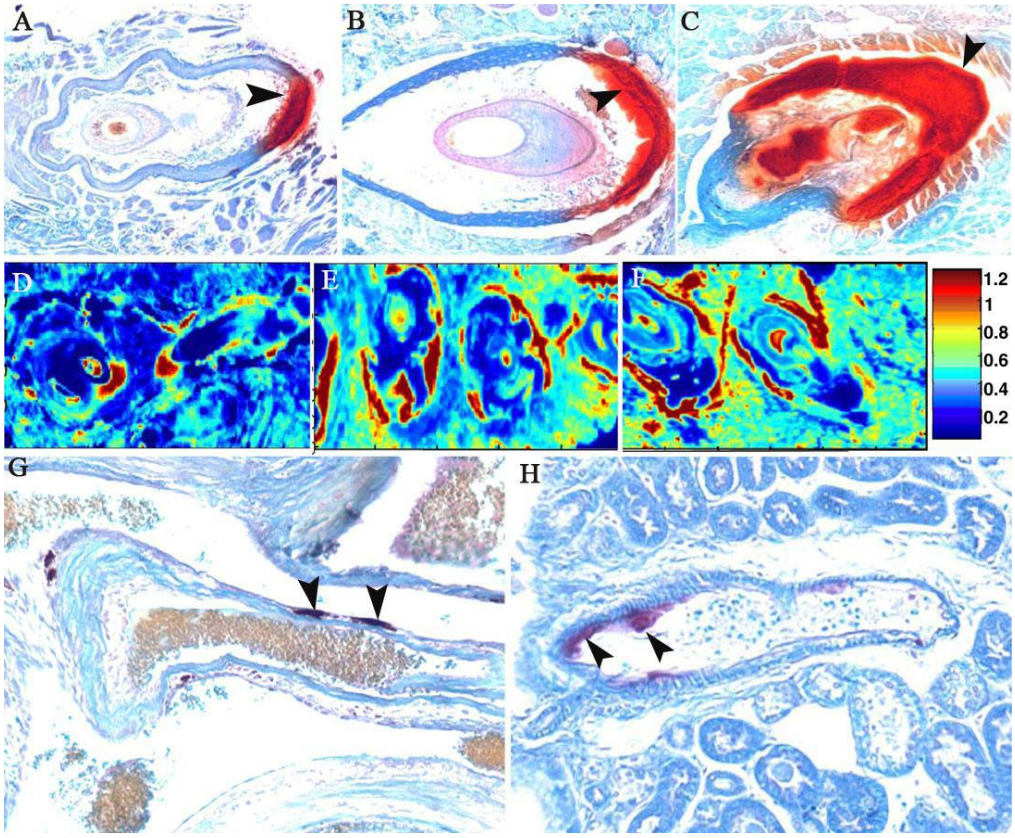

Fig. 5 Ectopic mineralization of the connective tissue capsule surrounding the vibrissae (A-C), and blood vessels in heart (G) and kidney (H), and FT-IRIS images of representative vibrissae samples from mice of different ages, based on the ratio of mineral to matrix peak heights at 1024 and 1660 cm^{-1}. Tissues were isolated from $Abcc6^{-/-}$ mice and processed for staining with Alizarin Red. Note the mineralization was indicated with open arrowheads. Note the progressive mineralization in the connective tissue capsule surrounding the vibrissae in 3 months (A, D), 6 months (B, E), and 24 months old mice (C, F), as well as mineralization in the blood vessels of heart and kidney at 24 months of age (G, H). The color scale represents the ratio of the heights of the absorbance bands of mineral to matrix where red indicates the highest and dark blue indicates the lowest (Kavukcuoglu et al., 2012).

Jiang and co-workers (2007) demonstrated that in these mice serum calcium and phosphate levels were normal although serum had less ability to prevent mineral deposition induced by inorganic phosphate in an *in vitro* system. Indeed, Fetuin-A, a potent inhibitor of calcification process, is reduced by 30% in the serum of $Abcc6^{-/-}$ mice compared to wild-type mice. The importance of Fetuin-A in preventing aberrant mineralization has been demonstrated by development of a mouse model in which the *Ahsg* gene was inactivated by targeted ablation. The $Ahsg^{-/-}$ mice developed severe calcification of various organs (Luo et al., 1997) and in particular Jiang et al. (2010) demonstrate that over-expression of Fetuin-A can prevent ectopic mineralization of connective tissues in PXE mouse model. Furhermore, another protein that is riduced in $Abcc6^{-/-}$ mice is Mgp, especially in the carboxilated form (that is the form active in inhibiting the calcification process) (Li et al., 2007).

The $Abcc6^{-/-}$ mouse has also been used to evaluate the effect of supplemented and/or modified diet on the mineralization process. In particular, to test if lowering the condition of chronic oxidative

stress can positively interfere with ectopic calcification (Li et al., 2008) mice were fed with antioxidants as vitamin C, vitamin E, selenium, and N-acetylcysteine. These diets were found to counteract oxidative stress, but failed to be effective on the mineralization process (Li et al., 2008). Other authors, to explore the potential efficacy of bisphosphonates to prevent ectopic calcification, fed Abcc6$^{-/-}$ mice with diet containing etidronate disodium (ETD) or alendronate sodium trihydrate (AST). They found that ETD, but not AST, significantly reduced mineralization, suggesting that selected bisphosphonates may be helpful for prevention of mineral deposits in PXE and GACI (Li et al., 2015). In addition, it is been demonstrated that also the mineral content of diet can modify the disease severity in PXE. For this reason a recent study explored the role of diet with reduced or increased magnesium in modifying tissue mineralization in mouse Abcc6$^{-/-}$. In 2010 Gorgels and coworkers demonstrated that increase in dietary magnesium reduces vascular calcification in a mouse model for PXE. Others authors, few years later, demonstrated an accelerated early-onset mineralization of connective tissues in mice Abcc6$^{-/-}$ with a diet in wich magnesium was reduced, suggesting that dietary magnesium levels may contribute to the phenotypic variability of PXE (Jiang and Uitto, 2012).

Generalized arterial calcification of infancy (GACI)

GACI is an early-onset, autosomal recessive disorder, which has only been described in approximately 100 mostly Caucasian patients (Li et al., 2012). GACI is caused by mutations in the ENPP1 gene, which encodes ectonucleotide pyrophosphatase/phosphodiesterase 1 (Rutsch et al., 2003). This enzyme hydrolyses ATP to AMP and inorganic pyrophosphate that serves as a powerful anti-mineralization factor. The disease typically affects children of less than 6 months of age (Rutsch et al. 2008; Moran, 1975). GACI is characterized by arterial stenosis, resulting from myointimal proliferation of muscular arteries, and early-onset severe myocardial ischemia due to extensive deposition of hydroxyapatite in the inner elastic lamina of medium- and large-sized arteries (Li et al 2012; Le Boulanger et al., 2010). Complications include myocardial infarction, hypertension and congestive heart failure, leading to early demise. The majority of patients die during the first year of life, with the highest fatality rate in the first six months of life, most commonly due to myocardial infarction, congestive heart failure, multiple organ failure or persistent arterial hypertension (Rutsch et al. 2008; Moran, 1975). Neonates with GACI can present rather aspecific symptoms, such as poor feeding and respiratory distress. Consequently the diagnosis is often only established by detecting arterial calcification using plain radiography, ultrasound or computed tomography. Typically, diffuse vascular and periarticular ectopic mineralization is found. Confirmation of the diagnosis is possible through molecular analysis of the *ENPP1* gene which

detects mutations in approximately 70% of cases (Rutsch et al. 2008; Rutsch et al., 2003; Ruf et al., 2005). The treatment options in GACI are limited and rely mostly on the use of bisphosphonates, such as etidronate and pamidronate. These bisphosphonates possibly act through decreasing bone turnover, further inhibiting growth of mineralized crystals and/or providing an alternative form of PPi thus influencing the regulation of mineralization process (Ramjan et al., 2009).

Keutel Sindrome

Since its first identification by Keutel *et al* in 1971, approximately 30 cases have been described of Keutel syndrome, which is an autosomal recessive multisystem disease with an age of onset in childhood (5-15 years) (Keutel et al., 1971; Weaver et al., 2014). Keutel syndrome is mainly characterized by peripheral pulmonary stenosis, abnormal cartilage ossification or calcification of typically (para)tracheal, bronchial and rib cartilages as well as auricular and nose cartilage (Khosroshahi et al., 2014). Less frequently soft tissue calcification, *i.e.*, of blood vessels, brain and kidneys, occurs (Vanakker et al., 2013). A long-term follow-up of 4 sisters with Keutel syndrome showed that all clinical manifestations were progressive. Nevertheless, the prognosis of Keutel syndrome is good in the most majority of patients, with life expectancy mainly depending on the severity of lung complications (Khosroshahi et al., 2014). Keutel syndrome is caused by loss-of-function mutations in the MGP gene, encoding matrix gla protein (Vanakker et al., 2013). MGP is an inhibitor of the pro-osteogenic BMP2-Smad-RUNX2 pathway, by inhibiting BMP2 binding to its receptor. Consequently, MGP expressed in chondrocytes functions as a local mineralization inhibitor under physiological conditions (Hale et al., 1988; Luo et al., 1997). Impairment of its inhibitory function favors pro-mineralizing signaling, leading to ectopic mineralization (Weaver et al., 2014). No etiologic treatment exists for Keutel syndrome, hence management is merely symptomatic, includes (angiographic) dilatation of peripheral artery stenosis and bronchodilating agents for respiratory symptoms (dyspnea and wheezing); the latter however can be inefficient in certain patients (Khosroshahi et al., 2014; Schurgers at al., 2007). Most patients develop hypertension before the age of 20, which can be treated with antihypertensive medication such as perindopril, amlodipine or nifedipine (Khosroshahi et al., 2014).

Idiopatic basal ganglia calcifictaion

Idiopathic basal ganglia calcification (IBGC) is a rare neurodegenerative disorder with unknown prevalence. The disease is sometimes referred to as Fahr's disease, although the patient Fahr had mineralization in blood vessels of the white matter of the brain (Moskowitz et al., 1971). IBGC affects young to middle aged adults, with an average onset in the 3rd or 4th decade of life; however

the disease has also been described in childhood (Kousseff et al., 1980; Saleem et al., 2013). IBGC is characterized by bilateral and (almost) symmetrical basal ganglia calcifications (Saleem et al., 2013). Ectopic mineralization may also occur in other brain regions, including the nucleus dentatus, thalamus, cerebral cortex and centrum semiovale (Saleem et al., 2013, Calabrò et al., 2014). Neurological symptoms include neuropsychiatric (cognitive impairment, depression, hallucinations, delusions, manic symptoms, anxiety, schizophrenia-like psychosis, personality changes) and movement disorders (*i.e*. parkinsonism, ataxia due to cerebellar involvement, tremor and paresis), as well as headache, vertigo, stroke-like events, orthostatic hypotension, dysarthria, seizures and papilledema due to raised intracranial pressure (Saleem et al., 2013; Dildar et al., 2014). Both sporadic and familial IBGC cases have been reported, the latter predominantly with autosomal dominant inheritance (Saleem et al., 2013). To date, mutations in 3 genes have been associated with IBGC, *i.e*., solute carrier family 20 (phosphate transporter) member 2 (*SLC20A2*), the beta polypeptide of platelet-derived growth factor (*PDGFB*) and platelet-derived growth factor receptor, beta (*PDGFRB*). So far, no genotype-phenotype correlation has been found (Wang et al., 2012). The *SLC20A2* gene, encoding a Pi transporter (also known as PiT2 which belongs to the type III sodium dependent phosphate transporter family), is expressed abundantly in a variety of tissues and likely plays a housekeeping role in cellular phosphate uptake (Wang et al., 2012; Mufaddel et al., 2014). Mutations in the gene have been described in more than 40 IBGC families worldwide and *in vitro* resulted in impaired Pi transport, leading to accumulation of this pro-mineralizing factor (Wang et al., 2012; Hsu et al., 2013; Westenberger et al., 2014). More recently, few IBGC patients were reported harboring mutations in *PDGFB* or *PDGFRB* (Nicolas et al., 2013; Keller et al., 2013). In animal models, *Pdgfrb* has been identified as an essential mediator in the development of pericytes in brain vessels, which have a key role in the maintening the blood-brain barrier (BBB). The BBB is hypothesized to be defective in IBGC (Nicolas et al., 2013). Moreover, Villa-Bellosta et al. (2011) found that the PDGFB-PDGFRB pathway seems to be involved in phosphate-induced calcifications in VSMCs by down regulating SLC20A2. All these data suggest that cerebral phosphate homeostasis plays a role in the development of vascular mineralization. The mineralization generally develops within the vessel wall and in the perivascular space, ultimately extending to the neuron. With time, calcification starts to compress the vessel lumen, which causes impaired blood flow, starting off a vicious circle with further neural tissue damage and mineral deposition. The mineral depositions tend to vary in composition according to their anatomical site and the proximity to vasculature calcifications, containing components such as calcium phosphate and carbonate; other compounds including glyconate, mucopolysaccharide and metals (iron, copper, magnesium, zinc, aluminum, silver and cobalt) may be also found (Saleem et al., 2013). IBGC

diagnosis is supported by the following criteria: (1) bilateral calcification of basal ganglia; (2) progressive neurologic dysfunction; (3) absence of biochemical abnormalities; (4) absence of infectious, traumatic or toxic cause; and (5) a significant family history (although sporadic IBGC cases have also been described) (Saleem et al., 2013).

Genetic testing can confirm the IBGC diagnosis. Sequencing of *SLC20A2* is the first choice, as well as deletion/duplication analysis if no mutation is found, with a mutation detection rate of 40%. If no mutations are found, *PDGFRB* and *PDGFB* sequencing can be performed; the precise mutation detection rate is currently unknown. If no molecular confirmation can be obtained, other (genetic) causes of brain calcification should be considered, before establishing a clinical diagnosis of IBGC (Saleem et al., 2013).

Arterial calcification due to CD73 deficency

Arterial calcification due to CD73 deficiency (ACDC), also referred to as calcifications of joints and arteries, is an autosomal recessive disease, which usually takes an onset in young adulthood (St Hilaire et al., 2011; Rutsch et al., 2011). ACDC is mainly characterized by prominent and often symptomatic calcification of the large arteries of the lower extremities (iliac, femoropopliteal and tibial arteries), typically sparing the coronary circulation (St Hilaire et al., 2011; Rutsch et al., 2011). The disease seems relatively rare, being only reported in 3 Caucasian families (Rutsch et al., 2011).

To elucidate the molecular etiology of ACDC, genomewide homozygosity mapping was performed in three families, revealing homozygous and compound heterozygous loss-of-function mutations in the *NT5E* gene (Rutsch et al., 2011; Martello et al., 2011).

NT5E encodes the glycosyl phosphatidylinositol (GPI)- linked plasma membrane CD73 ecto-enzyme, which has 5' ectonucleotidase activity and thus converts AMP to extracellular adenosine and Pi. The enzyme is located on the plasma membrane of vascular cells, supplying adenosine to cell surface receptors (Martello et al., 2011). Adenosine is produced immediately downstream of ENPP1 in the extracellular ATP-degradation pathway on the surface of vascular cells, and a lower adenosine level leads to impaired inhibition of TNAP (Rutsch et al., 2011; St Hilaire et al., 2011). St Hilaire *et al* (St Hilaire et al., 2011) hypothesized that increased TNAP activity reduces PPi levels, allowing calcification to occur. Because of the rarity of the disease, no treatment guidelines are available. Bisphosphonates, which were proven to be successful in GACI, possibly by restoring PPi levels, may also be a good treatment option in ACDC (Ramjan et al., 2009; St Hilaire et al., 2011). Other possible therapeutic options include adenosine receptor agonists or direct TNAP inhibitors (*e.g.*, lansoprazole), although further investigations are needed (St Hilaire et al., 2011).

Hyperphosphatemic familial tumoral calcinosis

Autosomal recessive hyperphosphatemic familial tumoral calcinosis (HFTC) is characterized by metastatic mineralization (Vanakker et al., 2013; Fathi et al., 2014). Patients usually show first signs in the first or second decade of life (Slavin et al., 1993).

The most prominent clinical manifestation of HFTC is periarticular mineralization of the skin and of subcutaneous tissue, mainly affecting the upper limbs and hip regions, although involvement of other localizations (spine, temporomandibular joints, metacarpals/metatarsals and popliteal space) have also been reported (Topaz et al., 2004). Calcium salt depositions usually present as firm painful tumor like masses, which may gradually enlarge over a period of years, causing functional problems including restricted joint mobility (Fathi et al., 2014; Topaz et al., 2004). Complications of the overlying skin, including pain, infection and ulceration, can cause scarring and deformity (Vanakker et al., 2013; Fathi et al., 2014; Topaz et al., 2004). Other possible manifestations of the disorder are dental abnormalities (Jose et al., 2010).

HFTC can be caused by mutations in UDP-N-acetylalpha- D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 3 (*GALNT3*), fibroblast growth factor 23 (*FGF23*) or klotho, all of which are key regulators of the phosphate metabolism (Vanakker et al., 2013).

Plain radiographs show the typical appearance of periarticular amorphous, multilobulated and cystic calcifications (Olsen et al.; 2006). A typical feature of HFTC is the absence of erosion/ bone destruction by adjacent soft-tissue masses (Fathi et al., 2014).

HFTC should be treated according to the location, size of the lesion and its relations to its environment. A first treatment option is medically reducing hyperphosphatemia through phosphate depletion, by dietary phosphorus restriction and/or the administration of phosphate binding chelating agents such as aluminum hydroxide.

A second treatment option is early surgical resection of the lesions. Alternative treatment options, include steroids, bisphosphonates, calcitonin and radiotherapy (Fathi et al., 2014).

Congenital deficiency of the vitamin K-dependent factors

Congenital deficiency of the vitamin K-dependent factors (VKCFD) is a very rare autosomal-recessive disorder. It is caused either by mutations in the GGCX gene (VKCFD1) on chromosome 2p12, or in the VKORC1 gene (VKCFD2) on chromosome 16p11.2. These genes encode a vitamin K-dependent carboxylase and vitamin K 2,3 epoxide reductase, respectively (Oldenburg et al., 2000; Li et al., 2004; Rost et al., 2004a, b; Zhang and Ginsburg, 2004). Both enzymes are essential for post-translational γ-carboxylation of clotting factors, enabling them to attach to the phospholipid bilayer of membranes as an essential prerequisite for blood coagulation (Zhang and Ginsburg,

2004). The clinical features of VKCFD-1 and -2 are highly variable and may include epistaxis, (neonatal) intracranial hemorrhage, hemarthrosis, etc., although several patients remain asymptomatic (Zhang and Ginsburg, 2004). Patients with this disorder might have an increased risk for cerebral aneurysms. By contrast, in PXE, cerebrovascular complications are mostly ischemic (stroke).

Chondrocalcinosis

Condhrocalcinosis is a condintion in wich there is a deposition of calcium crystals in the articular cartilage, with pain and artritis. There are different forms of chondrocalcinosis. Some may be linked to mutations of the ANKH gene (Pendleton et al., 2002) other forms (more frequently observed) are associated with aging (Ryan and McCarty, 1988). In subjects with ANKH mutations, protein activity it was not decreased but was increased with a consequent increase of extracellular PPi. In this context, PPi increases with cartilage aging (Rosenthal et al., 1994; Rosen et al., 1997; Rachow and Ryan, 1988), and these changes are strongly linked to idiopathic/sporadic chondrocalcinosis (Ryan and McCarty, 1988). Significantly, TGF-β potently induces increased extracellular PPi in articular chondrocytes, an activity that appears to be mediated by both TGF-β-induced PC-1 expression and PC1 translocation to the plasma membrane (Johnson et al., 1999; Lotz et al., 1995). Other mechanisms may also contribute to elevated PPi generation by aging cartilage, including increased extracellular availability of the NTPPPH substrate ATP (Ryan et al., 1991) and altered mitochondrial PPi metabolism in aging (Arthur PG et al., 1999), as illustrated by the observation that PPi concentration also increases in blood platelets in association with human aging (Ryan and McCarty, 1988).

4. PXE MODEL

Soft connective tissues calcification (i.e. ectopic calcifications) represent a deleterious consequence of diabetes, renal disorders and ageing, being a key determinant of cardiovascular morbidity and mortality. Molecular pathways leading to this undesired mineralization have been largely investigated in vascular smooth muscle cell cultures (VSMC) demonstrating that these cells undergo osteoblastic differentiation and have an active role in the mineralization process. Nevertheless, a key question is whether all mesenchymal cells have a similar behaviour, or if differences in their tissue-specific phenotype may be associated to the different susceptibility of connective tissues to mineralize. In particular, the skin is rarely affect by pathological mineralization in contrast to the frequent occorrence of ectcopic calcification in the cardiovascular system. Interestingly, there is a number of genetic disorders characterized by mineralization of the skin and of other soft connective tissues, indicating that all mesenchymal cells, in appropriate environments, mayexhibit a shift towards a pro-osteogenic phenotype possibly through similar pathways. Among these pathologic condition, *Pseudoxanthoma elasticum* (PXE) represent a paradigmstic model for investigating the pathogenesis of ectopic calcification.

To date, there are only few studies on the role of dermal fibroblasts in the mineralization process and therefore, in order to understand the contribution of these cells we have used the ***human PXE model***. The purpose of this study was to investigate wether dermal fibroblasts from PXE patients were more responsive to pro-calcifying stimuli compared to control cells from healthy subjects (Boraldi F, Annovi G, Bartolomeo A, Quaglino D. *J Dermatol Sci* 2014). Moreover, it is still unclear if changes occurring in fibroblasts' phenotype are the cause or the consequence of ectopic mineralization. Since the diagnosis of PXE can be posed only after the onset of clinical manifestations, i.e. after the ccurrence of calcification, the human PXE model cannot be used. Therefore, we have analysed fibroblasts obtained from $Abcc6^{+/+}$ and $Abcc6^{-/-}$ mice of 0.5 (absence of calcification) and 12 months of age (presence of mineral) (***animal PXE model***) (Boraldi F, Bartolomeo A, Li Q, Uitto J, Quaglino D. *J Invest Dermatol* 2014). It has to be underlined, in fact, that these mice develop aberrant connective tissue calcification only after 5-6 week of age and therefore it is possible to investigate fibroblasts already before the onset of pathologic calcification.

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Fibroblasts from patients affected by Pseudoxanthoma elasticum exhibit an altered PPi metabolism and are more responsive to pro-calcifying stimuli

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Pseudoxanthoma elasticum

ABSTRACT

Background: Pseudoxanthoma elasticum (PXE) is a genetic disorder characterized by progressive calcification of soft connective tissues. The pathogenesis is still hard to pin down. In PXE dermal fibroblasts, in addition to impaired carboxylation of the vitamin K-dependent inhibitor matrix Gla protein (MGP), we have also demonstrated an up-regulation of alkaline phosphatase activity. In the light of these data we have suggested that both calcium and phosphate metabolism might be locally altered, both pathways acting in synergy on the occurrence of matrix calcification.
Objective: This study aims to better explore if cultured PXE fibroblasts, compared to control cells, exhibit a modified inorganic pyrophosphate (PPi) metabolism and are more responsive to pro-calcifying stimuli.
Methods: Primary human dermal fibroblasts isolated from healthy individuals and from PXE patients were cultured for different time points in standard and in pro-calcifying media. The expression of *ANKH*/ANKH, *ENPP1*/PC1, *ALPL*/TNAP, *SPP1*/OPN was evaluated by qRT-PCR and Western blot, respectively. TNAP activity was measured by spectrophotometric analyses, whereas calcification was investigated by light and electron microscopy as well as by micro-analytical techniques.
Results: In the presence of pro-calcifying stimuli, dermal fibroblasts alter their phenotype favouring matrix mineralization. In particular, *ENPP1*/PC1 and *SPP1*/OPN expression, as well as TNAP activity, was differently expressed in control and in PXE fibroblasts. Moreover, in pathologic cells the ratio between factors favouring and reducing PPi availability exhibits a more pronounced shift towards a pro-calcifying balance.
Conclusion: PXE fibroblasts are more susceptible to pro-calcifying stimuli and in these cells an altered PPi metabolism contributes to matrix calcification.

1. Introduction

Ectopic calcification is a deleterious and worldwide complication affecting soft connective tissues [1]. In the last decade, the involvement of numerous genes/proteins has been disclosed in a number of investigations, indicating that pathologic mineralization is the result of an active and dynamic balance of pro- and anti-calcifying mechanisms [2,3]. Never the less, there are no therapeutic options capable to efficiently inhibit the occurrence of ectopic calcification [4], suggesting that at least some of the pathogenetic mechanisms are still elusive and need to be further explored.

Within this context, Pseudoxanthoma elasticum (PXE), an autosomal recessive disorder mainly affecting skin, eyes and the cardiovascular system, is typically characterized by a progressive mineralization of the elastic component of soft connective tissues [5] without any clear evidence of inflammation, cell necrosis or apoptosis [6]. Moreover, in PXE, in the absence of increased circulating levels of calcium and phosphate [7], calcified elastic fibres have a calcium/phosphate ratio similar to that of physiologically mineralized tissues as bone [8]. Surprisingly, PXE is associated to mutations in the ABCC6 gene [9] that encodes for a transmembrane protein highly expressed in liver and kidney, whereas it is barely detectable in soft connective tissues [6]. Clinical data and experimental observations indicate that, in PXE, ectopic calcification is not a passively diffused phenomenon, but

Abbreviations: *ALPL*/TNAP (*gene*/protein), tissue-nonspecific alkaline phosphatase isozyme; *ANKH*/ANKH (*gene*/protein), progressive ankylosis protein homolog; CM, calcifying medium; *ENPP1*/PC1 (*gene*/protein), ectonucleotide pyrophosphatase/phosphodiesterase family member 1; MGP, Matrix Gla Protein; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; PXE, Pseudoxanthoma elasticum; *SPP1*/OPN (*gene*/protein), osteopontin.

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affects peculiar matrix components in specific anatomical areas, although the pathogenetic mechanisms are still elusive. It has been suggested that elastic fibre calcification is the result of altered regulatory mechanisms that, possibly mediated or induced by factors abnormally released from the liver [6,10–12], may influence mesenchymal cells. These cells are responsible for triggering soft connective tissue mineralization in defined areas and not in the whole matrix, as it would occur simply due to the presence of altered circulating levels of calcium and phosphate and/or of other systemic factors [13–15]. At least in the skin, fibroblasts should be considered the principal candidates for the occurrence and progression of dermal ectopic calcification [16].

In previous *in vitro* studies, we have demonstrated that PXE fibroblasts are unable to properly carboxylate matrix Gla protein (MGP) [7,17,18], a potent vitamin K-dependent inhibitor of calcium deposition [19] and are characterized by increased alkaline phosphatase activity [7]. However, up-regulated TNAP activity is necessary, but not sufficient, to induce the mineralization process, as demonstrated in *in vitro* standard cell culture conditions [18]. An appropriate ratio between inducers and inhibitors of calcifications (either present in serum supplemented media or produced by cells) is in fact required to have hydroxyapatite actively deposited on the cellular monolayer [7,20].

Aim of this study was to investigate whether primary human dermal fibroblasts from PXE patients are more responsive to pro-calcifying stimuli than control cells from healthy individuals. Moreover, we have investigated if these fibroblasts exhibit a differential expression of genes/proteins involved in the inorganic pyrophosphate (PPi) metabolism (*i.e.* progressive ankylosis protein homolog, *ANKH*/ANKH; ectonucleotide pyrophosphatase/phosphodiesterase family member 1, *ENPP1*/PC1; tissue-nonspecific alkaline phosphatase isozyme, *ALPL*/TNAP). Moreover, given the regulatory role of PC1, ANKH and TNAP on osteopontin (*SPP1*/OPN) [21], the expression of this inhibitor of mineral crystal growth [22,23] was also investigated.

To address these questions, cells were cultured in standard (DMEM) and in calcifying media (CM) (*i.e.* in the absence/presence of calcifying stimuli, respectively) and evaluated at early and late time points (8 h and 20 days, respectively). These two time points were selected with the aim to investigate (i) if changes occurring in cells well before mineral deposition takes place could be indicative of a particular susceptibility of cells to pro-calcifying stimuli, and (ii) if the mineralizing environment provides a source of signalling cues that may further influence cell phenotype.

2. Materials and methods

2.1. Cells

Human dermal fibroblasts were available from the cell bank at the Cell-Lab "Paolo Buffa" (University of Modena and Reggio Emilia, Modena – Italy), being collected along the years in accordance with the guidelines of the ethical committee of the local Faculty of Medicine. Briefly, fibroblasts were isolated from skin biopsies, cultured in DMEM (Gibco, Grand Island, NY, USA) with 10% foetal bovine serum (FBS) (Lonza, Basel, CH), and stored in liquid nitrogen until use [24].

Cells were from 8 clinically healthy females (41 ± 8 years), who did not exhibit any sign of genetic, metabolic or connective tissue disorders, and from 10 patients affected by PXE (44 ± 11 years), as demonstrated by clinical and biomolecular (ABCC6 mutations) analyses.

During all experimental procedures, fibroblasts from each individual were kept separate and used between 3rd and 8th passages. In each experiment, control and pathologic cells were used at the same passage. Cells were routinely cultured in 75 cm^2 flasks (Nunc, Roskilde, Denmark) with DMEM supplemented with 10% FBS, penicillin 100 Ul/ml, streptomycin 100 μg/ml and non-essential aminoacids 1X (Gibco). At confluence, all fibroblasts were further cultured in standard medium (*i.e.* DMEM plus 10% FBS) (DMEM) or in a more complex calcifying medium (CM) (*i.e.* DMEM supplemented with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10 nM dexamethasone (Sigma–Aldrich, Milan, Italy) [7]. Media were replaced three times a week.

2.2. In vitro *calcification: von Kossa staining and analysis by environmental scanning electron microscopy (ESEM) and X-ray energy dispersive spectroscopy (EDS)*

Fibroblasts were routinely cultured in six-well plates (BD-Falcon, Franklin Lakes, NJ) up to confluence, when CM replaced the standard medium. At different time points, cells were fixed in 4% paraformaldehyde, stained with 2.5% silver nitrate, placed under a UV lamp for 30 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for two minutes. Von Kossa-positive (black) deposits were observed after alcohol washes. Mineralized areas were quantified using the Image J software. Experiments were conducted two times on all cell lines. At the same time, parallel cultures were fixed in methanol for 10 min and then samples were observed with a scanning electron microscope

Table 1
Primers used for real time polymerase chain reaction.

Gene	Positions	Sequence 5′–3′	Tm	%GC	Slope	*E*%	R^2
ALPL							
Forward	1044–1066	TACAAGCACTCCCACTTCATCTG	60	47	−3.353	98.7	0.915
Reverse	1108–1131	GCTCGAAGAGACCCAATAGGTAGT		50			
ANKH							
Forward	2268–2289	CACATGGCCGTACAAAGAGATG	60	50	−3.426	95.8	0.920
Reverse	2327–2351	GTGTGGAGTAGATGGTTTCGAACTC		48			
ENPP1							
Forward	2107–2129	CCGTGGACAGAAATGACAGTTTC	60	47	−3.207	105	0.989
Reverse	2166–2192	ATGGACAGGACTAAGAGGAATTCTAAA		37			
SPP1							
Forward	325–342	CTCCTAGCCCCACAGAAT	60	56	−3.432	95.6	0.955
Reverse	398–415	GGTCATGGCTTTCGTTGG		48			
CLK2							
Forward	1295–1313	GCACCATAGCACCATTGTC	60	52	−3.354	98.7	0.978
Reverse	1374–1394	GCAGCCTATACTCCACACATC		52			

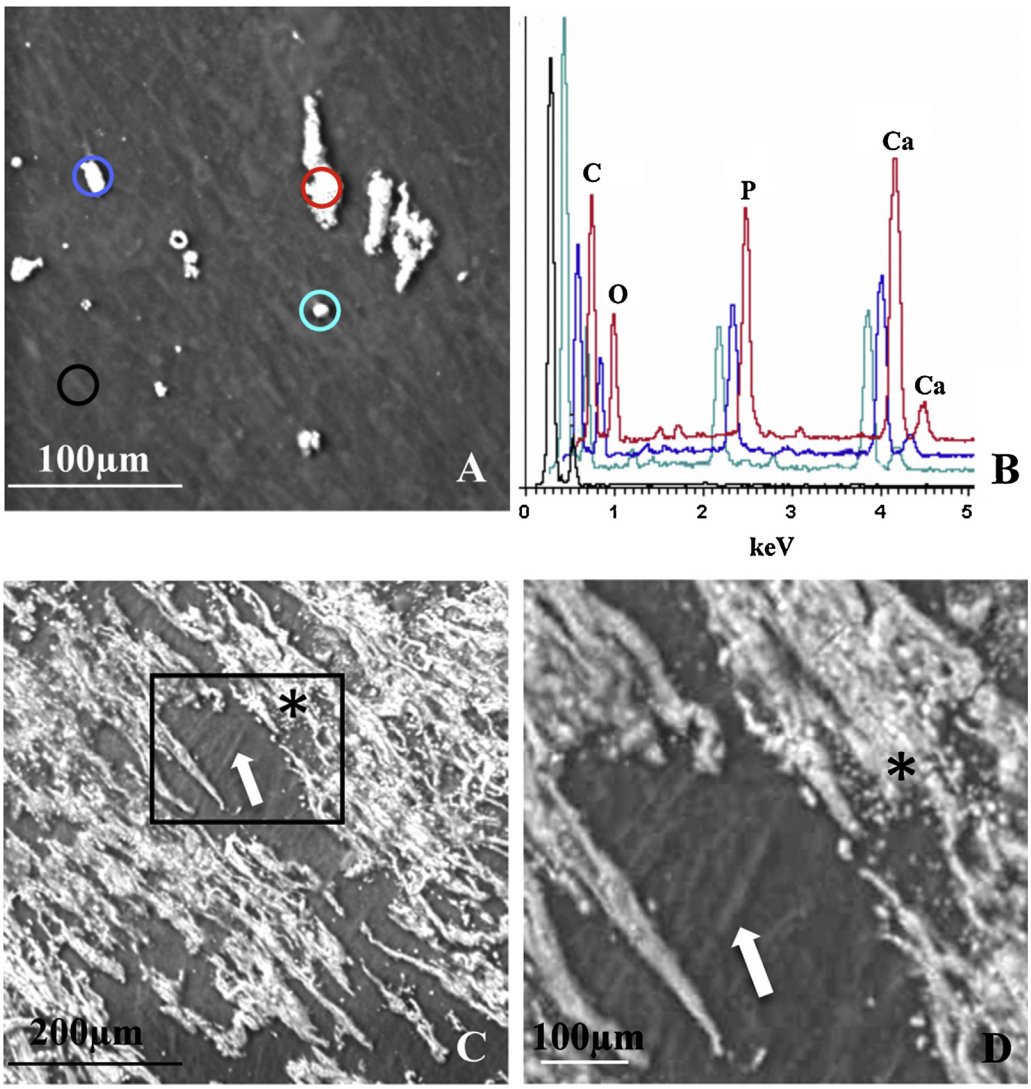

Fig. 1. Characterization of mineral deposits in dermal fibroblasts cultured in calcifying medium. Mineral deposits are visualized by ESEM on PXE fibroblasts after 20 (A) and 40 days (C and D). Panel D shows, at higher magnification, the distribution of hydroxyapatite bright deposits (*) on the surface of elongated fibroblasts (arrow). EDS spectra (B) demonstrate that mineral deposits are made of calcium (Ca) and phosphate (P), whose levels are proportional to the size/amount of calcification.

(FEI-ESEM Quanta 200) (FEI, Hillsboro, OR, USA) in Low vacuum mode. Solid state detector (SSD) for backscattered electrons and Large field detector (LFD) for secondary electrons were used for imaging. Microanalysis was performed using X-EDS (Oxford – INCA-350) (Oxford Instruments, Austin, TX, USA) [8].

2.3. Tissue-nonspecific alkaline phosphatase isozyme activity

Fibroblasts were detached by enzymatic treatment and washed three times with PBS. Cells were lysed by appropriate buffer containing 0.1% Triton X-100 (Sigma). Cell lysate (20 µL) was mixed with 100 µL Tris–glycine buffer pH 10.3 (50 mM Tris–HCl, 100 mM glycine and 2 mM $MgCl_2$) and 100 µL of p-nitrophenyl phosphate (Sigma). The reaction mix was incubated at 37 °C for 30 min and the reaction stopped by adding 50 µL of 3 M NaOH. Absorbance was measured at 405 nm in a microplate reader. Enzymatic activity was normalized to cell number. Control samples were set at 1. Experiments were performed at least three times in triplicate.

2.4. RNA preparation and quantitative real-time PCR (qRT-PCR)

Confluent cells were cultured in DMEM or in CM for a short (8 h, *i.e.* early CM) and a longer length of time (20 days, *i.e.* late CM). At each time point, total RNA was extracted using RNAeasy Protect cells Mini kit reagent (Qiagen, Valencia, CA) following manufacturer's instructions. Quality and quantity of RNA were checked with a spectrophotometer and by agarose gel electrophoresis. cDNA was then reverse-transcribed from 3 µg of total RNA using superscript III Reverse Transcriptase (Invitrogen, Monza, Italy). The amplification efficiency of primers was between 95% and 105% [25]. Primers and their characteristics are listed in Table 1. A 3× diluted cDNA sample was further amplified on an iCycler (BioRad, Segrate, Italy) using SYBR®-GreenER™qPCR SuperMix (Invitrogen), according to manufacturer's instructions. Thermal cycling parameters were set at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, an annealing temperature of 60 °C for 30 s, and of 72 °C for 15 s, followed by melting curve analysis with a temperature ranging from 95 °C to 55 °C.

Gene expression in each sample was normalized to the expression of a housekeeping gene (*CLK2*) that has been considered more suitable for determinations in dermal fibroblasts [26]. Data were evaluated using the Pfaffl method [27] based on real-time PCR efficiencies and compared with control cells. All qRT-PCR analyses were carried out in triplicate.

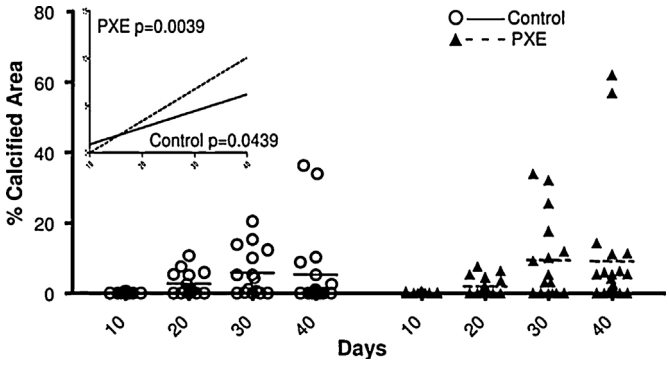

Fig. 2. Mineral deposition in control and PXE dermal fibroblasts cultured in calcifying medium. The extent of mineralization has been evaluated after von Kossa staining on control and PXE fibroblast cell cultures at different time points (from 10 up to 40 days). The percentage of calcified areas on the whole monolayer measured in each strain at same passages is shown. The increase of mineralization in control and PXE fibroblasts is time-dependent, as demonstrated by linear regression analysis (insert).

2.5. Protein extraction and Western blot (WB)

Cells were homogenized in RIPA buffer with protease inhibitors (Sigma), centrifuged and supernatants collected and stored at −80 °C until analysis. Protein concentration was measured in each sample according to Bradford assay [28] in order to load equal amounts of proteins. After separation by 1D PAGE [18], proteins were transferred to nitrocellulose and incubated with primary antibodies and thereafter with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, UK). The following primary antibodies against ANKH (Abcam), OPN (Abcam), PC1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNAP (Santa Cruz Biotechnology), βactin (Sigma) and S100A4 (Abcam) were used. Experiments were performed at least two times with all different cell lines.

2.6. Statistical analysis

Values were analyzed by Mann Whitney and by Anova test with significance at $p \leq 0.05$. Statistical data were obtained using GraphPad software 5.0 (San Diego, CA, USA).

3. Results

In order to induce mineralization *in vitro*, fibroblasts, as all other mesenchymal cells, must be cultured in a pro-calcifying medium that provides cells of the appropriate environment [20].

Mineral deposits were characterized by environmental scanning electron microscopy (ESEM) and by micro-analytical analyses. By ESEM, calcification appeared as small round precipitates that, with time in culture, coalesced into bigger areas of mineralization (Fig. 1A, C and D). However, even after 40 days of culture in CM, mineral deposits were not spread all over the cellular monolayer, but there were areas in which cells were devoid of mineral precipitates (Fig. 1C and D). EDS spectra (Fig. 1B) demonstrated that calcified areas were made of calcium and phosphate, whose concentration was proportional to the presence and to the size of precipitates (Fig. 1B), thus confirming that, although in an *in vitro* experimental model, the mineralization process mimic what takes place *in vivo* [8]. Comparable data were obtained on cell cultures from PXE patients (Fig. 1) and from control individuals (data not shown).

The extent of calcification was evaluated in control and PXE fibroblasts by light microscopy after the von Kossa staining (Fig. 2). As already demonstrated [7], cells cultured in DMEM never exhibited mineral deposition that, on the contrary, occurred in the presence of CM starting around day 20 and then progressively

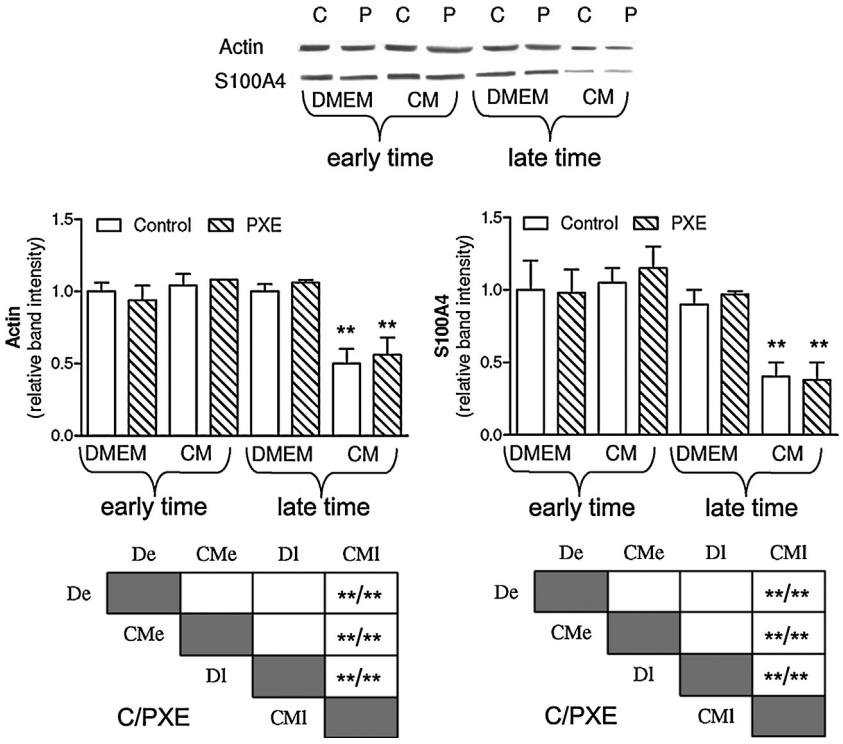

Fig. 3. Western blot for actin and S100A4 protein expression. Confluent cells were cultured for 8 h (early time) and 20 days (late time) in standard (DMEM) and in calcifying (CM) media. Control (C) and PXE (P) fibroblasts at same passages are compared. A representative Western blot is shown in upper panel, whereas mean values from all cells strains ± SE are shown in histograms. $^{**}p \leq 0.01$; De (early time point in DMEM), CMe (early time point in CM); DI (late time point in DMEM), CMI (late time point in CM).

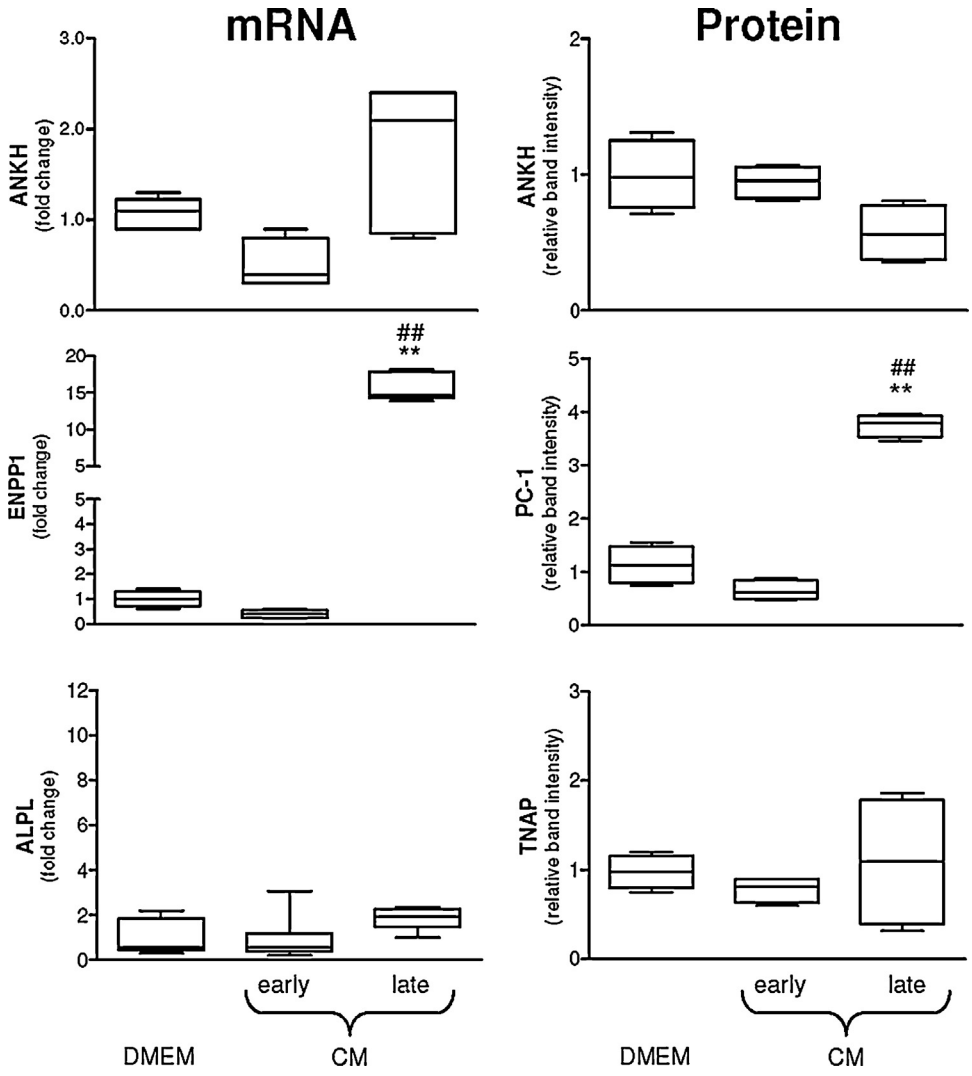

Fig. 4. PPi-related gene and protein expression in control fibroblasts. RNA and protein expression of progressive ankylosis protein homolog (*ANKH*/ANKH), ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (*ENPP1*/PC1) and of tissue-nonspecific alkaline phosphatase isozyme (*ALPL*/TNAP) were evaluated by RT-PCR and Western blot in control fibroblasts at confluence in standard medium (DMEM) (set at 1) and in calcifying medium (CM) for 8 h (early) and 20 days (late). Data from two experiments performed in triplicate with all fibroblasts cell lines are expressed as box-and-whisker plots showing the range of mRNA and protein expression values. The horizontal line shows the median value for each experimental condition, boxes represent the inter-quartile range and whiskers indicate the full extent of minimum/maximum values. $^{**}p \leq 0.01$ late *vs.* early times in CM; $\#\#p \leq 0.01$ CM *vs.* DMEM.

increased (Fig. 2). Crystal deposition was rather heterogeneous on the cellular monolayer with a variability being highly dependent on cell strain. Consistently, some PXE fibroblast cell strains exhibited a more extended surface area covered by mineral precipitates compared to control cells (Fig. 2).

It has been reported that culturing cells in CM affects mesenchymal cell phenotype by changing the expression of cytoskeletal and calcium binding proteins [29,30], actin and S100A4 were therefore analyzed by Western blot on fibroblasts cultured in DMEM and in CM at early and late time points (8 h and 20 days, respectively). Both actin and S100A4 were expressed in all culture conditions and in all cell strains. Interestingly, their expression significantly decreased after several days in CM (Fig. 3), demonstrating that these proteins were down-regulated when cells were actively involved in matrix calcification and that fibroblasts modified their phenotype in a mineralized environment.

Since we have demonstrated that TNAP activity is up-regulated in PXE, it was suggested that phosphate metabolism may be locally altered, thus contributing to the mineralization process [7]. Pyrophosphate-related gene and protein expressions were therefore investigated on control and PXE fibroblasts. Cells were cultured in a pro-calcifying environment in order to disclose, in an *in vitro* system, whether PXE compared to control fibroblasts were more responsive to mineralization stimuli.

No significant differences in the expression of *ANKH*/ANKH, *ENPP1*/PC1, *ALPL*/TNAP were detected at mRNA and protein levels in all cell strains cultured in DMEM (data not shown), in agreement with the inability of cells to calcify in these *in vitro* experimental conditions. By contrast, control fibroblasts cultured in CM at early and late time points revealed that, compared to basal levels of expression (*i.e.* confluent cells in DMEM), *ENPP1*/PC1 expression was significantly up-regulated at a late time point, when mineralization takes place (Fig. 4). Differences in *ANKH* and *ALPL* expression were negligible at all time points (Fig. 4).

A similar trend was observed also in PXE fibroblasts (Fig. 5), however, compared to basal levels, PC1 expression was significantly down- and up-regulated at early and late time points, respectively.

Comparison of control and PXE fibroblasts (Fig. 6) revealed that PXE cells, at the early time point in CM, had a significant down-regulation of PC1 expression, whereas values were similarly up-regulated in all cell strains at later time points (Fig. 6B). Differences

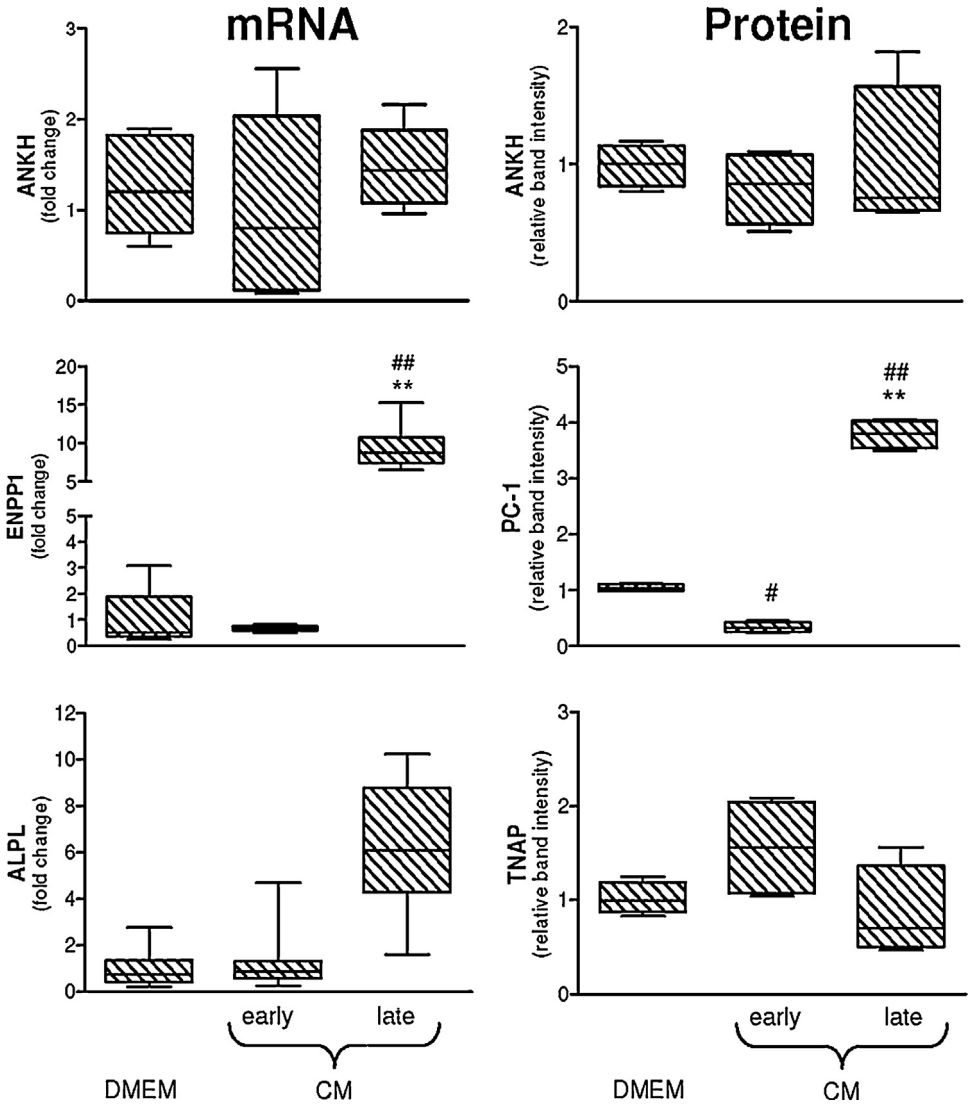

Fig. 5. PPi-related gene and protein expression in PXE fibroblasts. mRNA and protein expression of progressive ankylosis protein homolog (*ANKH*/ANKH), ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (*ENPP1*/PC1) and of alkaline phosphatase tissue-nonspecific isozyme (*ALPL*/TNAP) were evaluated by RT-PCR and Western blot in PXE fibroblasts at confluence in standard medium (DMEM) (set at 1) and in calcifying medium (CM) for 8 h (early) and 20 days (late). Data from two experiments performed in triplicate with all fibroblasts cell lines are expressed as box-and-whisker plots showing the range of mRNA and protein expression values. The horizontal line shows the median value for each experimental condition, boxes represent the inter-quartile range and whiskers indicate the full extent of minimum/maximum values. $^{**}p \leq 0.01$ late *vs.* early times in CM; $\#p \leq 0.05$, $\#\#p \leq 0.01$ CM *vs.* DMEM.

in ANKH expression were negligible in all experimental conditions (Fig. 6A). As already suggested [7], the mineralization process, at least *in vitro*, is associated to a progressive up-regulation of TNAP activity. In agreement with these observations, in the present study enzyme activity was progressively up-regulated in all cell strains (Fig. 6C), but values were significantly higher in PXE compared to control fibroblasts. Furthermore, since calcification is the results of a ratio between inducers and inhibitors of the mineralization process, including those regulating PPi, in Fig. 6D is shown the ratio between factors involved in PPi production and transport and factors that utilize PPi as a substrate for Pi release. This ratio, taken as an indicator of the mineralization potential of cells, appeared to progressively decrease as mineralization takes place in both cell lines. Interestingly, PXE cells have significantly lower values than control fibroblasts (Fig. 6D).

In the extracellular milieu, osteopontin acts as a calcification inhibitor whose expression can be modulated by phosphate and pyrophosphate availability and by TNAP activity [21,31,32]. *SPP1* expression was significantly down-regulated in control and PXE fibroblasts at early time point in CM (Fig. 7A and C). Moreover, OPN expression, at early time point, was significantly reduced in PXE compared to control cells (Fig. 7E), but it appeared increased up to basal levels in all cell strains at late compared to early time points (Fig. 7B and D).

4. Discussion

Pseudoxanthoma elasticum (PXE) is a genetic disease characterized by progressive ectopic calcification in soft connective tissues, including the skin [6]. In these patients, plasma levels of calcium and phosphate are normal, suggesting that other mechanisms are involved in the control of mineralization [7]. We have previously shown that, in the absence of any altered value in the circulation, TNAP activity is up-regulated in PXE fibroblasts and that levamisole, a specific TNAP inhibitor, suppresses mineralization *in vitro*. Therefore, it was hypothesized that in these cells, in addition to impaired MGP carboxylation, a modified PPi metabolism contributes to soft connective tissue calcification

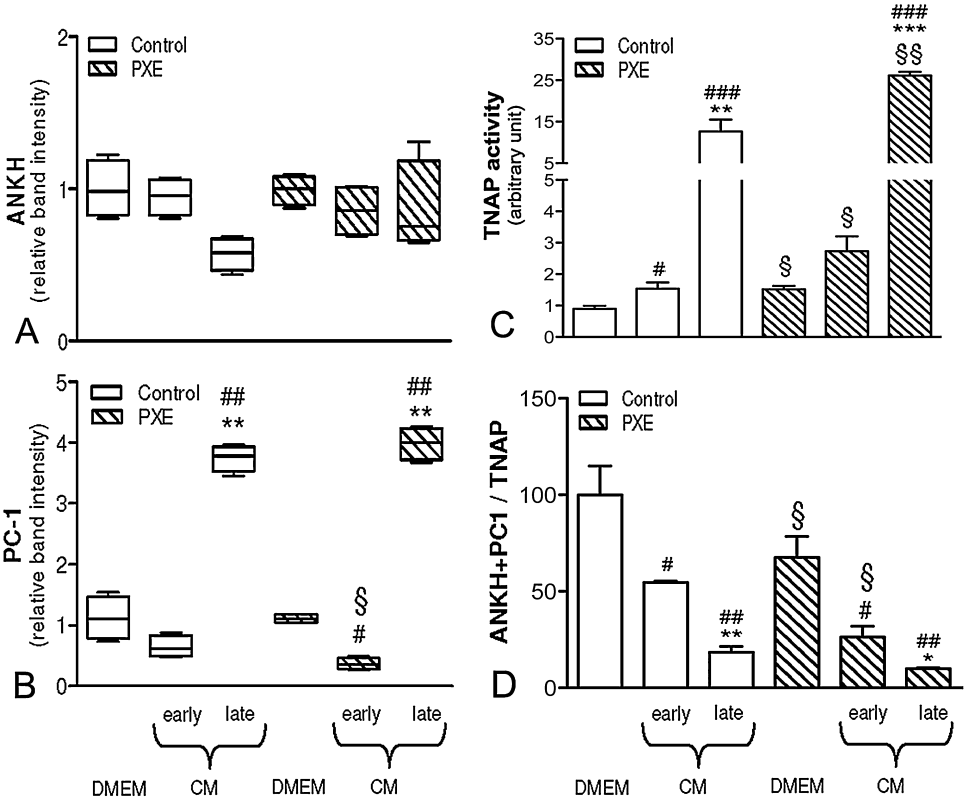

Fig. 6. PPi-related proteins in control and PXE fibroblasts. Control and PXE fibroblasts were cultured in standard medium (DMEM) (set at 1) and in calcifying medium (CM) for 8 h (early) and 20 days (late). Expression levels of progressive ankylosis protein homolog (ANKH) (A) and of ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (PC-1) (B), evaluated by Western blot, are expressed as box-and-whisker plots. The horizontal line shows the median value for each experimental condition, boxes represent the inter-quartile range and whiskers indicate the full extent of minimum/maximum values. TNAP activity (C) and the ratio (D) between factors increasing (PC1 and ANKH) and reducing (TNAP) PPi availability are expressed as mean values ± SE. All data are from two experiments performed in triplicate with all fibroblasts cell lines at same passages. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ late *vs.* early time point in CM; $\#p \leq 0.05$, $\#\#p \leq 0.01$, $\#\#\#p \leq 0.001$ CM *vs.* DMEM; $\S p \leq 0.05$, $\S\S p \leq 0.01$ PXE *vs.* Control in the same culture condition.

[7]. In agreement with this hypothesis, fibroblasts, being mesenchymal cells responsible for the correct amount and the appropriate quality of cellular and extracellular proteins, regulate the local balance of mineralization inducers and inhibitors and therefore can be advantageously used as an *in vitro* experimental model [16].

The present work aimed to study the effects of a pro-calcifying environment on control and PXE dermal fibroblasts by specifically looking at changes in the expression of genes and proteins involved in PPi metabolism. In particular, we have investigated the expression of PC1 that generates PPi from nucleoside triphosphates, of ANKH that mediates intracellular to extracellular PPi transport and of TNAP that hydrolyses PPi [21].

As already demonstrated, a standard cell culture environment is unsuitable to promote mineralization *in vitro*, independently from the ability of cells to induce calcification *in vivo* [33]. Consistently, in the present study, even after a long time of culture in DMEM, fibroblasts maintained their phenotype and mineralization never occurred. Moreover, the expression of mineralization-related genes/proteins regulating PPi metabolism exhibited negligible differences at various time points in culture as well as between control and PXE fibroblasts.

Therefore cells were cultured in an appropriate pro-calcifying environment that provides the stimuli necessary to induce the mineralization process *in vitro* [20,34]. In order to avoid the passive mineral deposition that, upon inorganic phosphate supplementation, was demonstrated *in vitro*, even without cells [35], a complex medium containing ascorbic acid, β-glycerophosphate and dexamethasone was used [7,20]. When cells were cultured in this pro-calcifying environment, there was a progressive hydroxyapatite deposition, even though mineralization was not homogeneously distributed over the monolayer. Calcification, in fact, is not a passive phenomenon and cells, even in the same environment, are actively and differently responsive to mineralization stimuli [20]. Present findings could explain why, in PXE, calcification occurs in specific areas in the absence of higher circulating levels of calcium and phosphate and without any evidence of inflammation or cell death.

The continuous presence of pro-calcifying stimuli is associated to cellular phenotypic changes [29,30]. In the present study, at late time point, fibroblasts reveal a cytoskeletal reorganization with reduced actin expression. Moreover, S100A4, a calcium-binding protein known to exert an inhibitory role on mineral deposition [29], was decreased to undetectable levels when the mineralized matrix is formed, indicating that calcification comes by changes in fibroblasts' behaviour.

Surprisingly, at early time point, before mineralization and before changes in fibroblasts' phenotype, PXE compared to control cells were characterized by significantly reduced expression of PC1 and increased TNAP activity. These changes act in synergy reducing the availability of PPi, a condition that represents a prerequisite for the mineralization process to take place [36]. Like PPi, OPN is a potent inhibitor of hydroxyapatite crystal growth [22,23]. Moreover, it has been demonstrated in osteoblasts that PPi is a specific signal for the induction of OPN expression [32] and that PPi deficiency in *ENPP1*$^{-/-}$ mice results in OPN deficiency [31]. In agreement with these observations a down-regulation of OPN has been demonstrated in PXE fibroblasts.

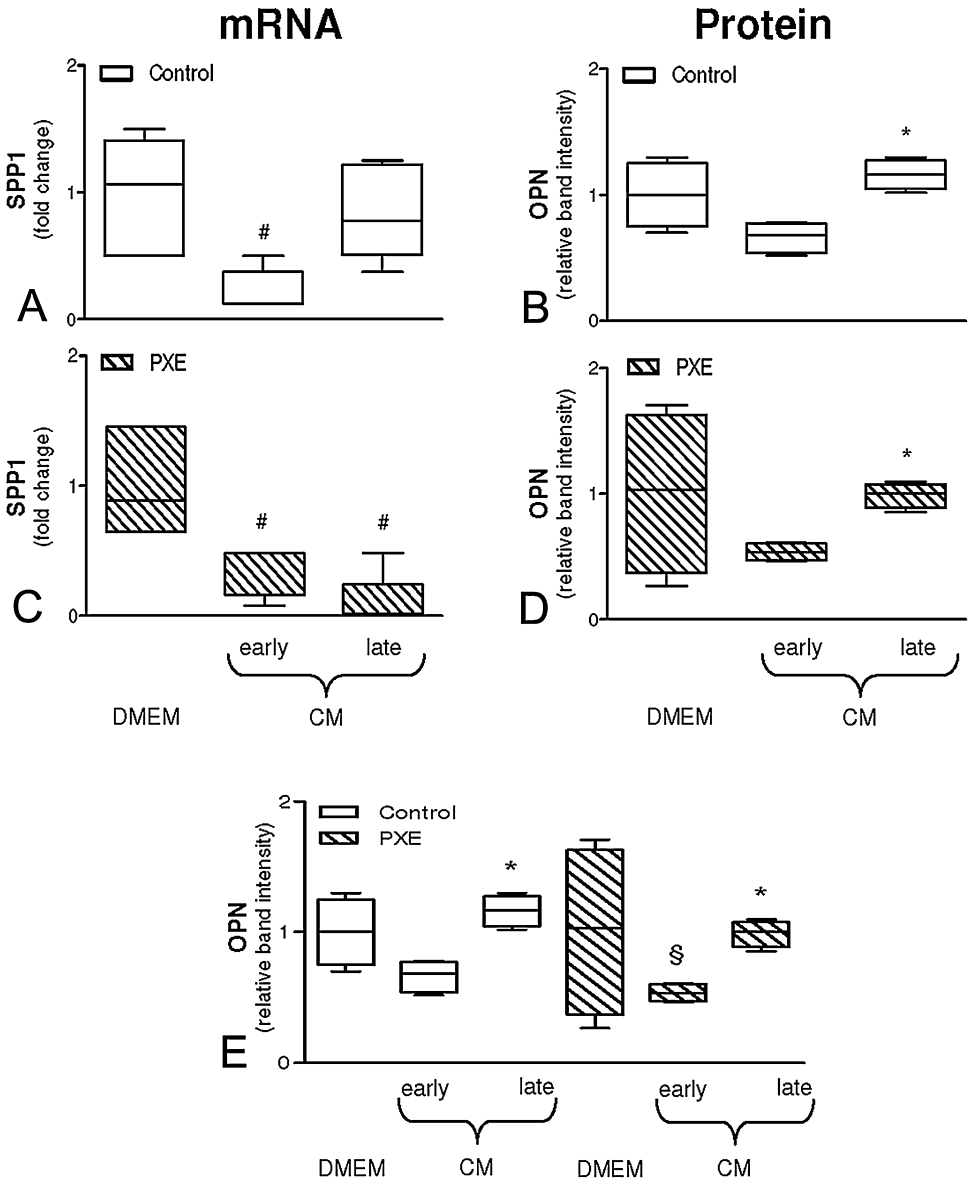

Fig. 7. Osteopontin gene and protein expression in control and PXE fibroblasts. mRNA and protein expression of osteopontin (*SPP1*/OPN) were evaluated by RT-PCR and Western blot in control (A and B) and in PXE (C and D) fibroblasts cultured in standard medium (DMEM) (set at 1) and in calcifying medium (CM) for 8 h (early) and 20 days (late). Comparison of OPN expression in control and in PXE fibroblasts at same passages is shown in panel E. Data from two experiments performed in triplicate with all fibroblast cell lines are expressed as box-and-whisker plots. The horizontal line shows the median value for each experimental condition, boxes represent the inter-quartile range and whiskers indicate the full extent of minimum/maximum values. $^{*}p \leq 0.05$ late *vs.* early time points in CM; $\#p \leq 0.05$ CM *vs.* DMEM; $\S p \leq 0.05$ PXE *vs.* Control in the same culture condition.

The altered expression of PPi-related proteins, as clearly shown at the early time point, indicates that PXE compared to control fibroblasts are particularly sensitive to a pro-calcifying environment.

At late time point, in a mineralizing environment, both control and PXE cells exhibited a significant up-regulation of *ENPP1*/PC1 as an attempt to counteract the mineralization process. However, PPi is a substrate for TNAP, whose activity was significantly up-regulated with time in CM, especially in PXE fibroblasts. After 20 days of culture, compared to the early time point in CM, OPN was up-regulated towards basal levels of expression.

It has to be noted that at late time point, after several days in the presence of pro-calcifying stimuli, the different response of control and PXE fibroblasts, observed at early time point, was no further detectable, being masked as a consequence of a secondary response of fibroblasts to the mineralized environment and/or of cumulative effects due to the extended permanence in CM.

We are aware that these culture conditions represent an artificial system, as are all *in vitro* models, since they should mimic in shorter times (few days or weeks) what takes place *in vivo* along the years under complex regulatory mechanisms active at both local and systemic levels. Never the less, results demonstrate that *in vitro*, if an adequate availability of calcifying stimuli are provided to cells, fibroblasts promote mineral deposition. These events are only partly dependent on TNAP activity, since enzyme up-regulation *per se* is not sufficient to favour the calcification process, as demonstrated for instance in cells cultured in standard medium [7], even in the presence of higher levels of enzyme activity. Never the less, even in PXE fibroblasts cultured in DMEM, the ratio between inducers and inhibitors of PPi metabolism is already shifted towards a pro-calcifying balance, suggesting that these cells may be more susceptible to mineralization stimuli compared to control fibroblasts. Mineralization takes place in all cell strains after several days in CM, when threshold levels of

calcification inducers exceed that of inhibitors. However, PXE fibroblasts, although with a variability depending on cell strain, showed higher amount of mineralization, supporting the hypothesis that these cells are more responsive to calcifying stimuli.

Moreover, it has to be underlined that in PXE, both *in vivo* and *in vitro*, the lower amount of carboxylated-MGP causes soft connective tissues being devoid of an appropriate and effective protection against mineral deposition [7,17,18]. Therefore, in PXE, both reduced MGP carboxylation and altered PPi metabolism may synergistically contribute to ectopic calcification.

In conclusion, mineralization may depend on fibroblast's subtype behaviour and responsiveness [37], on matrix composition (both in terms of quality and quantity of secreted proteins as well as on proteolytic activities) [6,38–40], on local calcium and phosphate availability [6] and on the ratio between mineralization inducers and inhibitors [7,16]. Results demonstrate that changes in PPi metabolism are present in PXE fibroblasts. Moreover, the altered balance between factors promoting or reducing PPi availability is already present in standard culture conditions, thus turning PXE fibroblasts into cells more responsive to pro-calcifying stimuli compared to controls. Therefore, we propose that circulating factors abnormally released in the circulation [10–12] are likely responsible for the development of a pro-calcifying environment that progressively induces responsive fibroblasts to alter their phenotype and to promote the mineralization process.

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Changes in Dermal Fibroblasts from Abcc6$^{-/-}$ Mice Are Present before and after the Onset of Ectopic Tissue Mineralization

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Pseudoxanthoma elasticum (PXE), a rare genetic disease caused by mutations in the *ABCC6* gene, is characterized by progressive calcification of elastic fibers in the skin, eyes, and the cardiovascular system. The pathomechanism of the mineralization is still obscure. Several hypotheses have been proposed, one of them suggesting a role for fibroblasts in controlling the amount and the quality of the calcified extracellular matrix. This hypothesis raises the question whether changes in mesenchymal cells are the cause and/or the consequences of the calcification process. In this study, fibroblasts were isolated and cultured from Abcc6$^{+/+}$ and Abcc6$^{-/-}$ mice of different ages to investigate parameters known to be associated with the phenotype of fibroblasts from PXE patients. Results demonstrate that a few changes (Ank and Opn downregulation) are already present before the occurrence of calcification. By contrast, a modification of other parameters (intracellular O_2^- content, Tnap activity, and Bmp2 upregulation) can be observed in Abcc6$^{-/-}$ mice after the onset of tissue mineralization. These data suggest that in the Abcc6$^{-/-}$ genotype, dermal fibroblasts actively contribute to changes that promote matrix calcification and that these cells can be further modulated with time by the calcified environment, thus contributing to the age-dependent progression of the disease.

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INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a hereditary disease caused primarily by mutations in the ABCC6 gene (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000), yet the mechanisms leading to elastic fiber mineralization and consequently to clinical manifestations are not well understood. A number of hypotheses have been proposed to explain how lack or impaired function of ABCC6, a transmembrane transporter protein almost exclusively expressed in the liver, can cause alterations in peripheral soft connective tissues, including elastic fiber calcification in the skin, eyes, and the arterial blood vessels (Quaglino *et al.*, 2011; Li *et al.*, 2012). It has been proposed that ABCC6 transporter deficiency may alter the availability of circulating factors, presumably metabolized and secreted by the liver, which are physiologically required to prevent aberrant calcifications and are able to modulate the phenotype of mesenchymal cells, such as fibroblasts (Ronchetti *et al.*, 2013). As a consequence of the abnormal protein profile of fibroblasts (Boraldi *et al.*, 2009), extracellular matrix components are differentially synthesized and/or degraded and elastic fiber mineralization ensues (Contri *et al.*, 1996; Passi *et al.*, 1996; Quaglino *et al.*, 2005; Gheduzzi *et al.*, 2007). Alternatively, it has been suggested that serum factors controlling soft connective tissue calcification are abnormally secreted by the liver and cause the calcification of elastic fibers. If this is the case, changes in the characteristics of mesenchymal cells could represent a secondary response to the mineralized environment (Le Saux *et al.*, 2006). A number of studies have been performed demonstrating that PXE fibroblasts, although isolated from patient tissues and cultured in an optimal *in vitro* environment, maintain a number of characteristics that clearly discriminate pathologic from healthy cells (Ronchetti *et al.*, 2013). Nevertheless, it is currently unclear what changes occur in fibroblasts before mineralization of elastic fibers and may be considered pathogenic for calcification, and what changes represent the cellular response to the mineralized environment. So far, all *in vitro* investigations on PXE have been performed on fibroblasts isolated and cultured from patients, i.e., from individuals who have been diagnosed on the basis of clinical manifestations as a consequence of calcification. Taking advantage of the PXE animal model, which recapitulates the PXE phenotype including the slow progression of tissue mineralization (Gorgels *et al.*, 2005;

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Abbreviations: ABCC6/Abcc6, ATP–binding cassette sub-family C member 6 (human/mouse); Ank, progressive ankylosis protein; Bmp2, bone morphogenic protein 2; Enpp1, pyrophosphatase/ phosphodiesterase 1; KO, knockout; Opn, osteopontin; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; PXE, pseudoxanthoma elasticum; ROS, reactive oxygen species; Tnap, tissue nonspecific alkaline phosphatase; WT, wild type

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Klement *et al.*, 2005), the aim of this study was to compare fibroblasts from congenic Abcc6$^{-/-}$ (knockout, KO) mice with those from Abc6$^{+/+}$ (wild type (WT)) mice. As calcification in these animals occurs only after 5–6 weeks of age (Klement *et al.*, 2005), fibroblasts were isolated from KO and WT mice at the age of 0.5 (absence of calcification) and 12 months (presence of calcification). A set of cellular parameters that we have previously shown to be altered in dermal fibroblasts from PXE patients and to be related to mineralization have been investigated (Boraldi *et al.*, 2009, 2013). Therefore, cells were characterized for their proliferative capabilities and for their redox balance (Quaglino *et al.*, 2000; Pasquali-Ronchetti *et al.*, 2006), on the basis of the intracellular content of reactive oxygen species (ROS). Moreover, the expression of proteins exerting a key role in the development of ectopic calcification (Giachelli 2005), such as bone morphogenic protein 2 (Bmp2), osteopontin (Opn), pyrophosphatase/phosphodiesterase 1 (Enpp1), progressive ankylosis protein (Ank), and tissue nonspecific alkaline phosphatase (Tnap), was evaluated by western blot analysis, and their Tnap activity (Boraldi *et al.*, 2013) was assessed by spectrophotometry.

RESULTS

Fibroblast proliferation capabilities

The number of fibroblasts that can be isolated from the skin of mice is progressively lower with advancing age, and therefore a higher number of adult animals was necessary to avoid differences in the number of population doublings of fibroblasts from younger and older animals during all experimental procedures. In contrast to human dermal fibroblasts, murine cells reach senescence around passage 4. Therefore, fibroblasts, independently from their genotype, were studied only at passages 2–3. Nevertheless, fibroblasts isolated from the skin of adult mice have a lower capacity to replicate *in vitro* compared with cells from younger animals and, when observed by phase contrast microscopy, appeared larger with cytoplasmic vacuoles (Figure 1a). Differences in the proliferation capabilities were independent from the genotype but were clearly related to the animal's age (Figure 1b).

Intracellular ROS content

As aging is characterized by an altered redox balance and an oxidative stress has been demonstrated in PXE either *in vitro* or *in vivo*, both in patients and in animal models (Pasquali-Ronchetti *et al.*, 2006; Garcia-Fernandez *et al.*, 2008; Li *et al.*, 2008), the intracellular ROS content was evaluated in mouse fibroblasts. Figure 2 shows that the H_2O_2 content is not affected by age or by the genotype. Interestingly, there was no difference in the amount of superoxide anion in fibroblasts from young WT and KO mice, whereas there was a significant increase in O_2^- in fibroblasts from adult Abcc6$^{-/-}$ mice compared with that in cells grown from WT animals of the same age or from younger Abcc6$^{-/-}$ mice (Figure 2).

Protein expression

No differences in Bmp2 expression were observed in young WT and KO animals (i.e., before the occurrence of calcification).

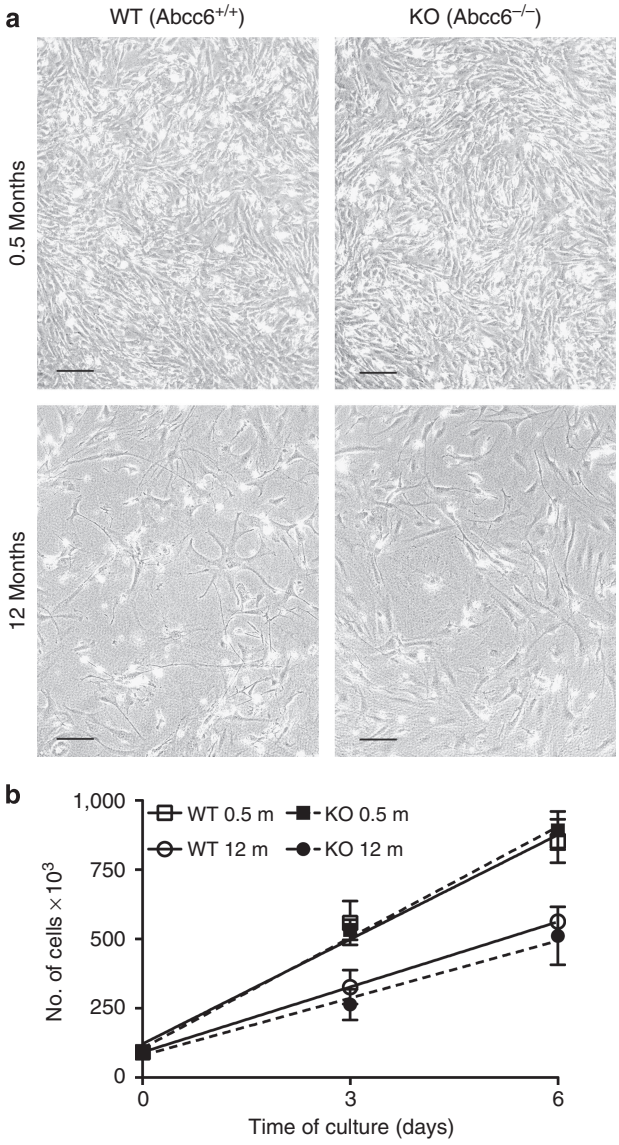

Figure 1. Dermal fibroblasts cultured from KO (Abcc6$^{-/-}$) and WT (Abcc6$^{+/+}$) mice of 0.5 and 12 months of age. Cells were observed by phase contrast microscopy (**a**) and counted after 3 and 6 days fropm plating (**b**). Differences in the proliferation capabilities are related to the age of the animals, but are independent of the genotype as clearly shown in graph (**b**). Bar = 300 µm. KO, knockout; WT, wild type.

With age, a significant upregulation was observed only in cells grown from adult KO animals (Figure 3).

Evaluation of Tnap protein expression revealed a significant increase with mice age, without differences between WT and KO animals (Figure 4). Similarly, Tnap activity was significantly upregulated in fibroblasts from adult mice compared with that in cells from younger mice (Figure 4). Moreover, enzymatic activity was even higher ($P<0.05$) in cells from adult KO mice compared with that in cells from WT mice of the same age (Figure 4).

In murine dermal fibroblasts, Enpp1 expression was significantly decreased in adult mice as compared with young animals; however, no differences were observed between Abcc6$^{-/-}$ and Abcc6$^{+/+}$ mice (Figure 5).

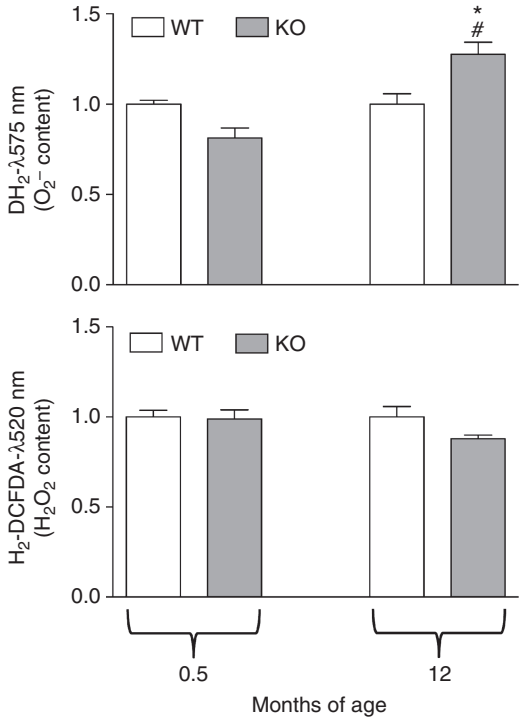

Figure 2. Anion superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content measured by flow cytometry in dermal fibroblasts cultured from WT (Abcc6$^{+/+}$) and KO (Abcc6$^{-/-}$) mice of 0.5 and 12 months of age. *$P<0.05$, KO vs. WT mice of the same age; #$P<0.05$, 0.5 vs. 12 months of age within the same genotype. KO, knockout; WT, wild type.

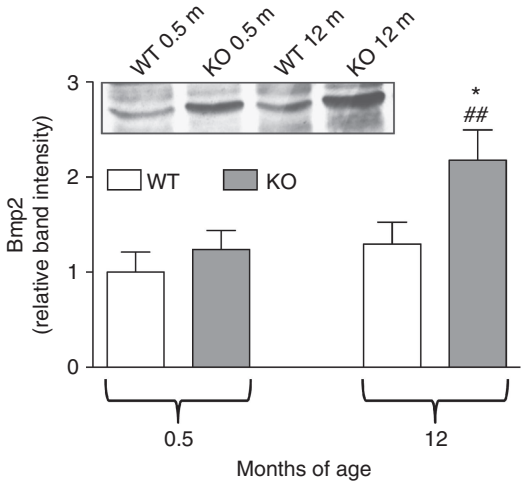

Figure 3. Expression of Bmp2 measured by western blot in dermal fibroblasts cultured from WT (Abcc6$^{+/+}$) and KO (Abcc6$^{-/-}$) mice of 0.5 and 12 months of age. Data are expressed as mean values ± SD of densitometric analyses, where values in cells from WT animals 0.5 months of age were set at 1. A representative western blot is shown. *$P<0.05$, KO versus WT mice of the same age; ##$P<0.01$, 0.5 vs. 12 months within the same genotype. KO, knockout; WT, wild type.

On the contrary, Abcc6$^{-/-}$ mice, at all ages, were characterized by a significantly lower expression of Ank compared with that in WT animals. However, no changes

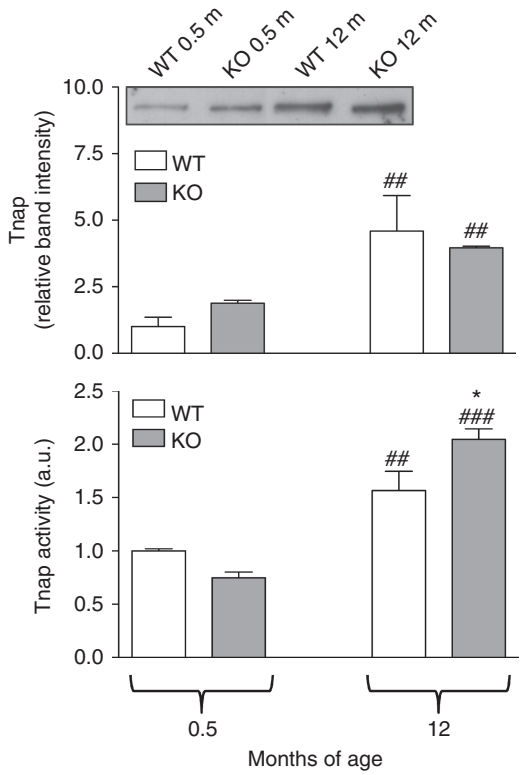

Figure 4. Tnap expression and activity were evaluated in dermal fibroblasts cultured from WT (Abcc6$^{+/+}$) and KO (Abcc6$^{-/-}$) mice of 0.5 and 12 months of age. Data are expressed as mean values ± SD of different measurements where values in cells from WT animals aged 0.5 months were set at 1. A representative western blot is shown. *$P<0.05$, KO versus WT mice of the same age; ##$P<0.01$ and ###$P<0.001$, 0.5 vs. 12 months within the same genotype. KO, knockout; WT, wild type.

were noted between cells from young and adult animals (Figure 5), indicating that this protein is not affected by the animal's age.

Osteopontin, a well-known inhibitor of the calcification process, was significantly reduced in Abcc6$^{-/-}$ fibroblasts compared with cells from age-matched WT mice. Differences were negligible with age (Figure 6), indicating that mouse genotype is the main regulator of Opn expression.

CONCLUSIONS

Although we have made significant progress in understanding the molecular basis of PXE, pathogenic mechanisms responsible for the calcification of elastic fibers in this disease remain to be clarified. Our understanding of PXE has been improved by the development of transgenic mouse models by specifically inactivating the *Abcc6* gene (Gorgels *et al.*, 2005; Klement *et al.*, 2005). Consistently, Abcc6$^{-/-}$ mice recapitulate several histopathological findings typical of PXE patients, including the slowly progressive mineralization of connective tissues in the skin, eyes, and the arterial blood vessels (Klement *et al.*, 2005; Jiang *et al.*, 2007). The aim of this study was to investigate fibroblasts isolated from the skin of Abcc6$^{-/-}$ and Abcc6$^{+/+}$ mice. Moreover, animals of two

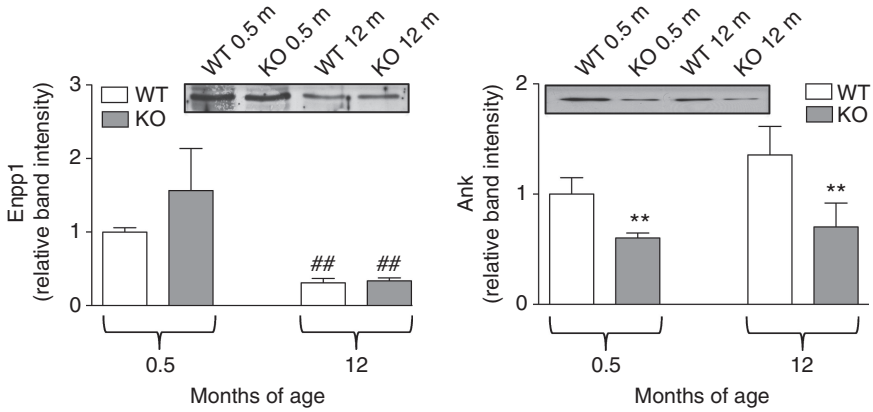

Figure 5. Pyrophosphatase/phosphodiesterase 1 (Enpp1) and ankylosis protein (Ank) expression measured by western blot in dermal fibroblasts cultured from WT (Abcc6$^{+/+}$) and KO (Abcc6$^{-/-}$) mice of 0.5 and 12 months of age. Data are expressed as mean values ± SD of densitometric analyses where values in cells from WT animals aged 0.5 months were set at 1. Representative western blots are shown. $^{**}P<0.01$ KO versus WT mice of the same age; $^{\#\#}P<0.01$, 0.5 vs. 12 months within the same genotype. KO, knockout; WT, wild type.

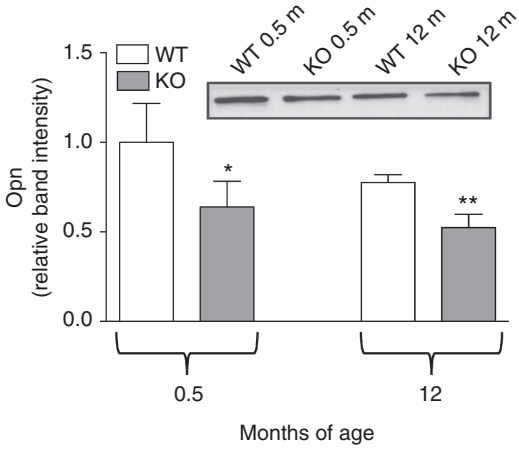

Figure 6. Expression of osteopontin (Opn) measured by western blot in dermal fibroblasts cultured from WT (Abcc6$^{+/+}$) and KO (Abcc6$^{-/-}$) mice of 0.5 and 12 months of age. Data are expressed as mean values ± SD of densitometric analyses where values in cells from WT animals aged 0.5 months were set at 1. A representative western blot is shown. $^{*}P<0.05$ and $^{**}P<0.01$ KO versus WT mice of the same age. KO, knockout; WT, wild type.

Table 1. Influence of genotype (WT vs. KO) and aging (0.5 vs. 12 months) on the expression of cellular parameters

	Changes influenced by	
Parameters	**Genotype**	**Age**
O_2: content	Yes	Yes
Bmp2: expression	Yes	Yes
Tnap		
Expression	No	Yes
Activity	Yes	Yes
Enpp1: expression	No	Yes
Ank: expression	Yes	No
Opn: expression	Yes	No

Abbreviations: AnK, progressive ankylosis protein; Bmp2, bone morphogenic protein 2; Enpp1, pyrophosphatase/phosphodiesterase 1; KO, knockout; O_2, oxygen; Opn, osteopontin; Tnap, tissue nonspecific alkaline phosphatase; WT, wild type.

different ages were used to compare the behavior of cells before with that after the onset of calcification. The present study design can identify changes that, when preceding ectopic mineralization, could contribute to the development of pathologic calcification, as well as differences that, when observed after the occurrence of mineralization, are possibly induced by the modified environment. To exclude changes simply related to the animal's aging, we have compared age-matched WT with KO mice (Table 1). The choice of this model was predicated on a number of reasons: (a) This mouse model allows the analysis of cells from tissues before animals with the Abcc6$^{-/-}$ genotype develop calcification, a condition that cannot be evaluated in humans as the histopathological diagnosis of PXE is routinely made after the onset of clinical manifestations, i.e., when the mineral precipitates are already present in tissues; (b) Fibroblasts are an excellent model for studying PXE, as they maintain an *in vitro* pathological phenotype (Quaglino *et al.*, 2000, 2005) exhibiting oxidative stress parameters (Pasquali-Ronchetti *et al.*, 2006) and an altered protein profile (Boraldi *et al.*, 2009, 2013). In this study, we have investigated the occurrence of oxidative stress and the expression of a number of proteins that are involved in the calcification process and, in particular, in the phosphate-related pathways, as we have hypothesized that they have a key role in the development and/or progression of soft connective tissue calcification in PXE (Li *et al.*, 2012, 2013; Boraldi *et al.*, 2013). Our results demonstrated that murine dermal fibroblasts, in contrast to human dermal fibroblasts (Boraldi *et al.*, 2010), exhibit

in vitro morphological features and proliferative capabilities that vary dependent on the animal's age and independently of the genotype. Therefore, the absence of the Abcc6 gene activity does not affect the proliferation potential of mesenchymal cells.

Replicative senescence in many *in vitro* models, including dermal fibroblasts, has been associated with increased ROS content (Lawless *et al.*, 2012). Altered redox balance is a frequent condition common to many disorders both in the absence or in the presence of ectopic calcification (Byon *et al.*, 2008), and has been demonstrated to be related to the severity of clinical manifestations in patients with PXE and PXE-like disorders (Garcia-Fernandez *et al.*, 2008; Boraldi *et al.*, 2013). As only fibroblasts from adult Abcc6$^{-/-}$ mice were shown to have higher levels of O_2^-, oxidative stress is likely the consequence of the calcified environment. Consistent with this conclusion, it has been demonstrated in the PXE animal model that antioxidant treatment can ameliorate the redox balance, without affecting the extent of mineralization (Li *et al.*, 2008).

Bmp2 expression is essential for osteoblastic differentiation of mesenchymal cells and therefore it has been widely implicated in the development of vascular calcification (Derwall *et al.*, 2012). Recent studies have shown that BMP2 is upregulated by ROS, being at the same time an inducer of ROS accumulation (Mandal *et al.*, 2011), and favoring alkaline phosphatase activity, and phosphate accumulation (Rawadi *et al.*, 2003). In accordance, we have shown that Bmp2, in association with ectopic calcification and with ROS accumulation, is significantly upregulated in fibroblasts from adult Abcc6$^{-/-}$ mice. Therefore, these data support the hypothesis that when calcification is initiated, there is a stimulatory loop that further increases mineral deposition and alters the phenotypic behavior of fibroblasts. A key question relates to the factors that are capable of triggering the initial phase of ectopic calcification in specific areas of soft connective tissues. We have already demonstrated that Tnap activity is significantly upregulated in fibroblasts from PXE patients, suggesting that phosphate metabolism has a role in the pathogenesis of elastic fiber calcification (Boraldi *et al.*, 2013). Present data further indicate that there is an age-dependent upregulation of Tnap expression and Tnap activity in fibroblasts from Abcc6$^{-/-}$ mice. Alkaline phosphatase acts on phosphate-containing substrates by releasing inorganic phosphate that can accumulate in the extracellular matrix and, under favorable conditions of the microenvironment, can precipitate in the form of hydroxyapatite crystals. One of these substrates is the calcification inhibitor inorganic pyrophosphate (PPi), which is generated by Enpp1 through hydrolysis of adenosine triphosphate and is transported to the extracellular milieu by Ank. Assessment of the expression of these proteins indicated that both the age and the genotype of mice contribute to phenotypic changes of the fibroblasts.

In particular, our data demonstrated that, at all time points, Abcc6$^{-/-}$ fibroblasts have a lower expression of Ank, a protein that is necessary for providing the extracellular environment with adequate amounts of physiological calcification inhibitors, including PPi. When Ank is downregulated, soft connective tissue becomes increasingly prone to mineralization. As this process gradually progresses with increased age of the animals, fibroblasts are modified toward a phenotype that favors mineral precipitation (i.e., lower Enpp1 and increased Tnap expression and activity). These observations are in agreement with the concept that ectopic calcification is more frequently observed in aged individuals.

Interestingly, besides PPi, other factors control mineral deposition in the extracellular matrix, including osteopontin, a well-known inhibitor of vascular calcification (Scatena *et al.*, 2007), as it regulates apatite crystal size and growth most likely by its ability to directly bind to specific apatite crystal faces (Speer *et al.*, 2002). The marked downregulation of Opn expression observed in fibroblasts from Abcc6$^{-/-}$ mice at all ages, together with lower PPi availability and a progressive Tnap-mediated release of Pi from the PPi pool in older mice, may act in synergy to promote mineral deposition.

In this study, a comparison of a number of parameters, before and after the onset of mineral deposition in tissues of PXE mice, demonstrated that there are changes in dermal fibroblasts that can be related to the genotype and that are present well before the development of calcification. Such changes possibly represent a trigger of calcification, whereas other changes may reflect a cellular response to the calcified environment that further sustains the mineralization process. Finally, the observation that Ank expression is significantly reduced in fibroblasts from the young Abcc6$^{-/-}$ mice supports the hypothesis that phosphate metabolism is locally altered in PXE (Boraldi *et al.*, 2013) and that reduced transport of the calcification inhibitor PPi in the extracellular compartment together with impaired carboxylation of Matrix Gla Protein (Gheduzzi *et al.*, 2007; Li et al., 2007; Boraldi *et al.*, 2013) may be the key players for the initial deposition of mineral precipitates in the absence of Abcc6 transport activity.

In conclusion, our data demonstrate that, although grown in optimal culture conditions, dermal fibroblasts from KO mice, similar to human PXE fibroblasts, exhibit and maintain *in vitro* peculiar characteristics. Therefore, at least few of the differences in protein expression are related to the genotype, whereas other parameters are modulated both by the genotype and by the age of the animals (Table 1). Although it cannot be excluded that circulating factors *in vivo* exert an epigenetic modulation on the fibroblast phenotype, the present data support the role of mesenchymal cells, such as fibroblasts in the mineralization process. These cells exhibit a differential expression of molecules related to the calcification process before ectopic calcification takes place within tissues, thus excluding the hypothesis that these changes are the consequence of the mineralized environment. Within this context, Ank and Opn may represent two potential pharmacologic targets warranting further investigation in the search for novel therapies for PXE.

MATERIALS AND METHODS

Cells

Dermal fibroblast cultures were obtained from the whole skin of 37 Abcc6$^{-/-}$ and 29 Abcc6$^{+/+}$ mice of 0.5 ($n=27$) and 12 months ($n=39$) of age. The study was approved by the Ethical Committee of

the University of Modena and Reggio Emilia. Fibroblasts were cultured in DMEM (Gibco, Grand Island, New York, NY) with 10% fetal bovine serum (Lonza, Basel, CH) and were used at 2nd or 3rd passages. During all experimental procedures, fibroblasts were pooled from several animals of the same age and genotype. Cells were routinely cultured in 75 cm^2 flasks (Nunc, Roskilde, Denmark) with DMEM supplemented with 10% fetal bovine serum, penicillin 100 IU ml^{-1}, streptomycin 100 μg ml^{-1}, and nonessential amino acids 1X (Gibco). Cells were observed by phase contrast microscopy.

Flow cytometry

ROS analysis was performed on proliferating cells as previously described (Boraldi *et al.*, 2009). Briefly, cells were stained with dihydroethidium (DH_2, 1 mM) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 2 mM) probes (Molecular Probes, Eugene, OR) for the determination of superoxide anion O_2^- and hydrogen peroxide (H_2O_2), respectively. Cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Brea, CA) at the emission wavelengths of 575 and 520 nm. Ten thousand events were collected and evaluated for each treatment using a WINMDI 2.8 program. Experiments were performed in duplicate and repeated twice.

Western blot

Cells were homogenized in RIPA buffer with protease inhibitors (Sigma, St Louis, MO), centrifuged, and supernatants collected and stored at −80 °C until analysis. Protein concentration was measured in each sample according to Bradford assay (Bradford, 1976) to load equal amounts of protein. After separation by 1D PAGE, proteins were transferred to nitrocellulose and incubated with anti-Ank, anti-Opn, anti-Bmp2 (Abcam, Cambridge, UK); anti-Enpp1 and anti-Tnap (Santa Cruz Biotechnology, Santa Cruz, CA), followed by appropriate horseradish peroxidase–conjugated secondary antibodies (Abcam). Experiments were performed at least two times with all different cell lines.

Alkaline phosphatase activity

Fibroblasts were detached by enzymatic treatment and washed three times with PBS. Cells were lysed by appropriate buffer containing 0.1% Triton X-100 (Sigma). Cell lysate (20 μl) was mixed with 100 μl of Tris-glycine buffer, pH 10.3 (50 mM Tris-HCl, 100 mM glycine, and 2 mM $MgCl_2$), and 100 μl of p-nitrophenyl phosphate (Sigma). The reaction mix was incubated at 37 °C for 30 min and the reaction was stopped by adding 50 μl of 3 M NaOH. Absorbance was measured at 405 nm in a microplate reader. Enzymatic activity was normalized to total protein concentration using BSA as a standard for the Bradford protein detection method (Bradford, 1976). Control samples were set as one. Experiments were performed three times in duplicate.

Data analysis

Data were expressed as mean values ± SD of all measurements and were compared by the Mann–Whitney or by the Anova test with significance at $P<0.05$. Statistical data were obtained using GraphPad software 5.0 (San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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5. Aging

Aging is a very complex physiological process, that causes a decline in the functions of the whole organism. It is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability up to death. This deterioration is the primary risk factor for major human pathologies including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases.

Aging research has experienced an unprecedented advance over recent years, particularly with the discovery that the rate of aging is controlled, at least to some extent, by genetic pathways and biochemical processes conserved in evolution. So a recent study proposes nine candidate hallmarks that are generally considered to contribute to the aging process and to the aging phenotype (López-Otín et al., 2013) (Figure 6).

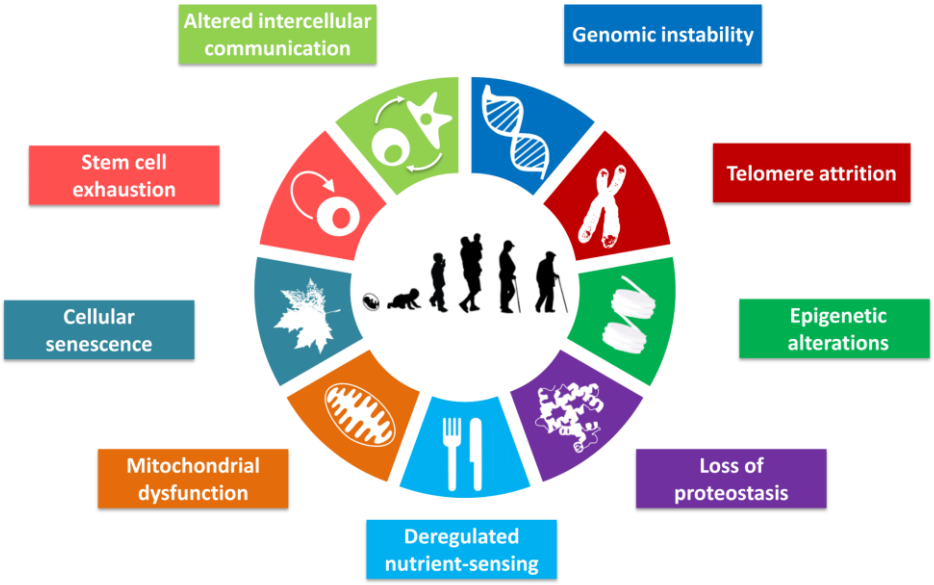


Fig. 6. The Hallmarks of Aging. The scheme enumerates the nine hallmarks described in a recent review: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín et al., 2013).

Genomic instability

Numerous premature aging diseases, such as Werner syndrome and Bloom syndrome, are the consequence of increased DNA damage accumulation (Burtner and Kennedy, 2010), although the relevance of these and other progeroid syndromes to normal aging remains unresolved due in part to the fact that they recapitulate only some aspects of aging. The integrity and stability of DNA is continuously challenged by exogenous physical, chemical and biological agents, as well as by endogenous threats including DNA replication errors, spontaneous hydrolytic reactions, and reactive oxygen species (ROS) (Hoeijmakers, 2009). The genetic lesions arising from extrinsic or intrinsic damage are highly diverse and include point mutations, translocations, chromosomal gains

and losses, telomere shortening, and gene disruption caused by the integration of viruses or transposons. To minimize these lesions, organisms have evolved a complex network of DNA repair mechanisms that are collectively capable of dealing with most of the damages inflicted to nuclear DNA (Lord and Ashworth, 2012). The genomic stability systems also include specific mechanisms for maintaining the appropriate length and functionality of telomeres, and for ensuring the integrity of mitochondrial DNA (mtDNA) (Blackburn et al., 2006; Kazak et al., 2012).

Somatic mutations accumulate within cells from aged humans and model organisms (Moskalev et al., 2012). Other forms of DNA damage, such as chromosomal aneuploidies and copy-number variations have also been found associated with aging (Faggioli et al., 2012; Forsberg et al., 2012). Increased clonal mosaicism for large chromosomal anomalies has been also reported (Jacobs et al., 2012; Laurie et al., 2012). All these forms of DNA alterations may affect essential genes and transcriptional pathways, resulting in dysfunctional cells that, if not eliminated by apoptosis or senescence, may jeopardize tissue and organismal homeostasis. This is especially relevant when DNA damage impacts on the functional competence of stem cells, thus compromising their role in tissue renewal (Jones and Rando, 2011).

Mutations and deletions in aged mtDNA may also contribute to aging (Park and Larsson, 2011). mtDNA has been considered a major target for aging-associated somatic mutations due to the oxidative microenvironment of the mitochondria, the lack of protective histones in the mtDNA, and the limited efficiency of the mtDNA repair mechanisms compared to those of nuclear DNA (Linnane et al., 1989). The causal implication of mtDNA mutations in aging has been controversial because of the multiplicity of mitochondrial genomes, which allows for the co-existence of mutant and wild-type genomes within the same cell, a phenomenon that is referred to as ‘heteroplasmy’.

The first evidence that mtDNA damage might be important for aging and age-related diseases derived from the identification of human multisystem disorders caused by mtDNA mutations that partially phenocopy aging (Wallace, 2005). Further causative evidence comes from studies on mice deficient in mitochondrial DNA polymerase γ. These mutant mice exhibit aspects of premature aging and reduced lifespan in association with the accumulation of random point mutations and deletions in mtDNA (Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008). Moreover, stem cells from these progeroid mice are particularly sensitive to the accumulation of mtDNA mutations (Ahlqvist et al., 2012).

In addition to genomic damage affecting nuclear or mtDNA, defects in the nuclear lamina can also cause genome instability (Dechat et al., 2008). Alterations of the nuclear lamina and production of an aberrant prelamin A isoform called progerin have also been detected during normal human aging (Ragnauth et al., 2010; Scaffidi and Misteli, 2006). The causal relevance of nuclear lamina

abnormalities in premature aging has been supported by the observation that decreasing prelamin A or progerin levels delays the onset of progeroid features and extends lifespan in mouse models of HGPS.

Telomere attrition

Accumulation of DNA damage with age appears to affect the genome near-to-randomly, but there are some chromosomal regions, such as telomeres, that are particularly susceptible to age-related deterioration (Blackburn et al., 2006). Replicative DNA polymerases lack the capacity to replicate completely the terminal ends of linear DNA molecules, a function that is proprietary of a specialized DNA polymerase known as telomerase.

Telomere exhaustion explains the limited proliferative capacity of some types of *in vitro* cultured cells, the so-called replicative senescence or Hayflick limit (Hayflick and Moorhead, 1961; Olovnikov, 1996). Not only telomeres are progressively shortened in the absence of telomerase but, also, even in the presence of telomerase, the infliction of exogenous DNA damage to telomeres becomes invisible to the DNA repair machineries due to the presence of shelterins. Therefore, DNA damage at telomeres causes a persistent type of DNA damage that leads to deleterious cellular effects including senescence and/or apoptosis (Fumagalli et al., 2012; Hewitt et al., 2012). Telomerase deficiency in humans is associated with premature development of diseases, such as pulmonary fibrosis, dyskeratosis congenita and aplastic anemia, which involve the loss of the regenerative capacity of different tissues (Armanios and Blackburn, 2012). In humans, recent meta-analyses have indicated a strong relation between short telomeres and mortality risk, particularly at younger ages (Boonekamp et al., 2013).

Epigenetic alterations

A variety of epigenetic alterations affects all cells and tissues throughout life (Talens et al., 2012). Epigenetic changes involve alterations in DNA methylation patterns, post-translational modification of histones, and chromatin remodeling. Increased histone H4K16 acetylation, H4K20 trimethylation or H3K4 trimethylation, as well as decreased H3K9 methylation or H3K27 trimethylation, constitute age-associated epigenetic marks (Fraga and Esteller, 2007; Han and Brunet, 2012). The multiple enzymatic systems assuring the generation and maintenance of epigenetic patterns include DNA methyltransferases, histone acetylases, deacetylases, methylases and demethylases, as well as protein complexes implicated in chromatin remodeling.

Loss of proteosatsis

Aging and some aging-related diseases are linked to impaired protein homeostasis or proteostasis (Powers et al., 2009). All cells take advantage of an array of quality control mechanisms to preserve the stability and functionality of their proteomes. Proteostasis involves mechanisms for the

stabilization of correctly folded proteins, most prominently the heat-shock family of proteins, and mechanisms for the degradation of proteins by the proteasome or the lysosome (Hartl et al., 2011; Koga et al., 2011; Mizushima et al., 2008). Moreover, there are regulators of age-related proteotoxicity, such as MOAG-4, that act through an alternative pathway distinct from molecular chaperones and proteases (van Ham et al., 2010). All these systems function in a coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins. Accordingly, many studies have demonstrated that proteostasis is altered with aging (Koga et al., 2011). Additionally, chronic expression of unfolded, misfolded or aggregated proteins contributes to the development of some age-related pathologies, such as Alzheimer's disease, Parkinson's disease and cataracts (Powers et al., 2009).

Deregulated Nutrient-sensing

The somatotrophic axis in mammals comprises the growth hormone (GH), produced by the anterior pituitary, and its secondary mediator, the insulin-like growth factor (IGF-1), produced in response to GH by many cell types, most notably hepatocytes. The intracellular signaling pathway of IGF-1 is the same as that elicited by insulin, allowing cells to respond to the presence of glucose. Remarkably, 'the insulin and IGF-1 signaling' (IIS) pathway is the most conserved aging-controlling pathway in evolution and among its multiple targets are the FOXO family of transcription factors and the mTOR complexes, which are also involved in aging and conserved through evolution (Barzilai et al., 2012; Fontana et al., 2010; Kenyon, 2010). Genetic polymorphisms or mutations that reduce the functions of GH, IGF-1 receptor, insulin receptor or downstream intracellular effectors such as AKT, mTOR and FOXO, have been linked to longevity, both in humans and in model organisms, further illustrating the major impact of trophic and bioenergetic pathways on longevity (Barzilai et al., 2012; Fontana et al., 2010; Kenyon, 2010). Consistent with the relevance of deregulated nutrient-sensing as a hallmark of aging, dietary restriction increases lifespan or healthspan in all investigated eukaryote species, including unicellular and multicellular organisms of several distinct phyla, including nonhuman primates (Colman et al., 2009; Fontana et al., 2010; Mattison et al., 2012).

Mitochondrial Dysfunction

As cells and organisms age, the efficacy of the respiratory chain tends to diminish, thus increasing electron leakage and reducing ATP generation (Green et al., 2011). The relation between mitochondrial dysfunction and aging has been long suspected but dissecting its details remains as a major challenge for aging research.

The mitochondrial free radical theory of aging proposes that the progressive mitochondrial dysfunction that occurs with aging results in increased production of ROS (Harman, 1965). Other mechanisms causing defective bioenergetics include accumulation of mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of the macromolecular organization of respiratory chain (super)complexes, changes in the lipid composition of mitochondrial membranes, alterations in mitochondrial dynamics resulting from imbalance of fission and fusion events, and defective quality control by mitophagy, an organelle-specific form of macroautophagy that targets deficient mitochondria for proteolytic degradation (Wang and Klionsky, 2011). Other mechanisms causing defective bioenergetics include accumulation of mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of the macromolecular organization of respiratory chain (super)complexes, changes in the lipid composition of mitochondrial membranes, alterations in mitochondrial dynamics resulting from imbalance of fission and fusion events, and defective quality control by mitophagy, an organelle-specific form of macroautophagy that targets deficient mitochondria for proteolytic degradation (Wang and Klionsky, 2011). The combination of increased damage and reduced turnover in mitochondria, due to lower biogenesis and reduced clearance, may contribute to the aging process.

Cellular Senescence

Cellular senescence can be defined as a stable arrest of the cell cycle coupled to stereotyped phenotypic changes (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007; Kuilman et al., 2010). This phenomenon was originally described by Hayflick in human fibroblasts serially passaged in culture (Hayflick and Moorhead, 1961). Today, we know that the senescence observed by Hayflick is caused by telomere shortening (Bodnar et al., 1998), but there are other aging-associated stimuli that trigger senescence independently of this telomeric process. Most notably, non-telomeric DNA damage and de-repression of the *INK4*/*ARF* locus, both of which progressively occur with chronological aging, are also capable of inducing senescence (Collado et al., 2007). The accumulation of senescent cells in aged tissues has been often inferred using surrogate markers such as DNA damage. Some studies have directly used senescence-associated β-galactosidase (SABG) to identify senescence in tissues (Dimri et al., 1995).

Since the amount of senescent cells increases with aging, it has been widely assumed that senescence contributes to aging. However, this view undervalues what conceivably is the primary purpose of senescence, which is to prevent the propagation of damaged cells and to trigger their demise by the immune system. Therefore, it is possible that senescence is a beneficial compensatory response that contributes to rid tissues from damaged and potentially oncogenic cells. This cellular checkpoint, however, requires an efficient cell replacement system that involves clearance of

senescent cells and mobilization of progenitors to re-establish cell numbers. In aged organisms, this turnover system may become inefficient or may exhaust the regenerative capacity of progenitor cells, eventually resulting in the accumulation of senescent cells that may aggravate the damage and contribute to aging. In recent years, it has been appreciated that senescent cells manifest dramatic alterations in their secretome, which is particularly enriched in pro-inflammatory cytokines and matrix metalloproteinases (Kuilman et al., 2010; Rodier and Campisi, 2011). This pro-inflammatory secretome may contribute to aging.

Stem Cell Exhaustion

The decline in the regenerative potential of tissues is one of the most obvious characteristics of aging. Although deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, an excessive proliferation of stem and progenitor cells can also be deleterious by accelerating the exhaustion of stem cell niches. Stem cell exhaustion unfolds as the integrative consequence of multiple types of aging associated damages and likely constitutes one of the ultimate culprits of tissue and organismal aging. Recent promising studies suggest that stem cell rejuvenation may reverse the aging phenotype at the organismal level (Rando and Chang, 2012).

Altered Intercellular Communication

Aging also involves changes at the level of intercellular communication, (Laplante and Sabatini, 2012; Rando and Chang, 2012; Russell and Kahn, 2007; Zhang et al., 2013). A prominent aging-associated alteration in intercellular communication is 'inflammaging' that may result from multiple causes such as the accumulation of pro-inflammatory tissue damage, the failure of an ever more dysfunctional immune system to effectively clear pathogens and dysfunctional host cells, the propensity of senescent cells to secrete pro-inflammatory cytokines. Inflammation is also involved in the pathogenesis of obesity and diabetes type 2, two conditions that contribute and correlate with aging in the human population (Barzilai et al., 2012). Likewise, defective inflammatory responses play a critical role in atherosclerosis (Tabas, 2010).

Beyond inflammation, accumulating evidence indicates that age-related changes in one tissue can lead to aging-specific deterioration of other tissues, explaining the inter-organ coordination of the aging phenotype. In addition to inflammatory cytokines, there are other examples of 'contagious aging' or bystander effects in which senescent cells induce senescence in neighboring cells via gap junction-mediated cell-cell contacts and processes involving ROS (Nelson et al., 2012). There are several possibilities for restoring defective intercellular communication underlying aging processes, including genetic, nutritional or pharmacological interventions that may improve the cell-cell

communication properties that are lost with aging (Freije and Lopez- Otin, 2012; Rando and Chang, 2012).

5.1 Models for ageing studying

It is generally accepted that aging has two principal determinants: the intrinsic disposition (genetic make up, somatic capacity and composition) delineating what is maximally possible, and extrinsic factors (life style, nutrition, environmental influences) determining how the pre-set frame of opportunity is exploited in the course of the individual ageing trajectory (Tigges et al., 2014).
Several studies have analyzed cellular behavior during physiological ageing with interesting, but often contradictory results, depending on the model used.
To investigate the mechanisms on the basis of ageing it is possible to use different models.
Cultured fibroblasts represent a widespread *in vitro* model for exploring the importance of specific molecular pathways leading to differentiation, ageing and death (Holbrook and Byers, 1989; Van Gansen and Van Lerberghe, 1987).
It has been reported that fibroblasts, *in vitro*, start to develop progressive morphological changes shortly after cultures are established, regardless of the donor's age (Robbins et al., 1970), but also that cell cultures from old and young donors exhibit structural and metabolic differences quantitatively and qualitatively distinct from those observed at early and late passages. It has been therefore suggested that *in vitro* ageing may represent a suitable system for examining the loss of replicative potential, whereas fibroblasts derived from adult/old and neonatal/young donors (*ex vivo* aging model), cultured up to replicative senescence (*in vitro* ageing model), may be considered an appropriate cellular ageing model.
Fibroblasts from aged donors (*ex vivo* ageing), already at early CPD, exhibit an impaired redox balance, highlighting the importance of this parameter during ageing, even in the presence of standard environmental conditions, which are considered optimal for cell growth (Boraldi et al., 2010).
Fisher *et al* (1996) suggested that in aged fibroblasts, UV radiations stimulate the expression and secretion of matrix metalloproteinases (MMPs) via upregulation of the transcription factors AP-1 and NF-kappa B. Enhanced expression MMP1 and decreased expression of the endogenous inhibitor TIMP-1 is the main cause for the degeneration of the extracellular matrix in extrinsically aged skin (Brennan et al., 2003)
Cellular senescence *in vitro* is obtained inducing telomere shortening, through continuous replication (Hayflick, 1980; Hayflick and Moorhead, 1961). A different type of cellular senescence can be induced in human diploid fibroblasts by oxidative stress or suboptimal cell culture

conditions (*i.e*. stress induced premature senescence, SIPS), which is independent of telomere shortening (Dierick et al., 2002; Toussaint et al., 2002, 2000).

Several proteins, as those related to heat shock response, or involved in endoplasmic reticulum and membrane trafficking, appeared differentially expressed only during *in vitro* ageing, suggesting that, with cumulative population doublings (CPD), the whole cell machinery becomes permanently altered. Farthermore, given the importance of the elastic component for a long-lasting connective tissue structural and functional compliance, it has been demonstrated a close relationship between fibulin-5 (FBLN5) and ageing.

Fibulin-5 represents a very sensitive ageing marker and could play an important role in EMC homeostasis, controlling also the redox balance. It is markedly decreased in the aged reticular dermis. FBLN5 is known to bind tropoelastin, to promote its coacervation (Hirai et al., 2007) and to induce elastic fiber assembly (Nakamura et al., 2002; Yanagisawa et al., 2002). Therefore, reduced FBLN5 expression could contribute to the reduced and less organized deposition of elastin aggregates observed in old fibroblasts already at low CPD. It should finally be noted that enhanced secretion of metalloproteinases is a feature of the altered secretory phenotype acquired in conjunction with cellular senescence. Furthermore, since dermal fibroblasts only rarely proliferate *in vivo*, it remains to be seen whether chromosomal instability observed in replicative fibroblast senescence *in vitro* reflects the situation in dermal fibroblasts in aged human skin.

As already mentioned, of great interest for the comprehension of aging, are the progeroid syndromes. These conditions are characterized by premature aging and a reduction in life expectancy due to genetic mutations. Children affected by these syndromes show different characteristics similar to those usually seen in older individuals. These include baldness, osteoporosis, dry and wrinkled skin.

Examples of these diseases are Werner syndrome, Wiedemann-Rautenstrauch syndrome, Hutchinson-Gilford syndrome and Down syndrome.

Werner syndrome produces scleroderma skin changes hair loss, premature onset of cataracts, muscle atrophy, glucose intolerance, high incidence of cancers. The involved gene in Werner syndrome encodes a DNA helicase (Epstein et a., 1966).

Even the Wiedemann-Rautenstrauch and Hutchinson-Gilford syndromes produce premature scleroderma, baldness and other age-related complications. The genetic basis of these syndromes remains undetermined (Arboleda et al., 2007).

Down syndrome mainly involves the central nervous system, generally resulting, in delay and acceleration of the formation of neuritic plaques and neurofibrillary tangles characteristic of Alzheimer's disease (Wilcock et al., 2015).

However, remain undetermined the specific genes involved in the prematurely aged-phenotype.

5.2 Aging and calcification

Aging is also associated to increased soft connective tissue mineralization, namely ectopic calcification as it is frequently observed in atherosclerosis, diabetes, arthritis and cardiovascular deseases (Li, et al., 2013).

Changes in the characteristics of the extracellular matrix and in the ratio between matrix constituents, influence not only the mechanical properties of connective tissues, but significantly contribute to modulate cell phenotype by altering integrin expression, focal adhesions, cytoskeletal organization and consequently intracellular signaling pathways. Therefore, beside alterations in the balance between pro- and anti-calcifying factors, changes in the extracellular matrix may significantly contribute to mineral deposition (Pugashetti et al., 2011).

In this contest, mesenchymal cells, play a major role being responsible for the production of most of the extracellular factors, capable to inhibit the mineralization process.

Vascular calcification, for istance, is characterized by an increase of the thickness of the vessel and loss of elasticity which causes an increase in speed of arterial pulsation and pressure, resulting in ventricular hypertrophy. Hypertension, diabetes and hyperlipidemia are risk factors for vascular calcification associated with cardiovascular events. Specific factors are likely to drive the calcification process, and to influence vascular smooth muscle cells, which are the predominant cell type involved in vascular calcification, undergoing a phenotypic transition to osteoblastic and osteocytic cells (Speer et al., 2005; Zhu et al., 2011).

Recent studies have demonstrated that low serum klotho is associated with signs of vascular dysfunction, such as vascular calcification (Kuro-o et al., 2006). Soluble klotho ameliorates vascular calcification by enhancing phosphaturia, preserving glomerular filtration and directly inhibiting phosphate uptake by vascular smooth muscle. Circulating klotho functions as a hormone that prevents vascular calcification. The klotho gene has been identified as an aging suppressor that encodes a protein involved in cardiovascular disease (CVD). Polymorphisms on this gene are associated in human with various events such as hypertension, diabetes and hyperlipidemia all risk factors for vascular calcification and cardiovascular events (Hong et al., 2015).

In contrast to intracellular proteins that are continuously synthesized and recycled, ECM proteins, in general, and elastic fiber conponents, in particular, are remarkably long-lived. In both small mammals and humans, elastic fiber proteins within the tissue are required to perform their mechanical role, for the entire lifetime of the organism (Shapiro et al., 1991). Specifically, during the course of 70 years, human aortic elastic fibers must undergo over 3 billion extension and recoil

cycles without repair or replacement. As a consequence, these assemblies are vulnerable to the accumulation of damages, and although the key cause of elastic fiber degradation remain to be determined, potential degradative mechanisms include glucose-mediated cross-linking, calcium and lipid accumulation, the time-dependent modification of aspartic acid residues, reactive oxygen species-mediated oxidation, and enzymatic proteolysis by the large family of matrix metalloproteinases. These proteases are expressed both constitutively and as a consequence of age related inflammatory conditions such as emphysema, atherosclerosis, and skin photoaging (Sherratt, 2009). The aging process has a profound effect on the structure of the elastic system and in areas exposed to UVR. The exposure of skin to low-dose UVR is sufficient to induce the expression and/or activation of MMP- 1, -2, -3, -7, -9, -12, and -13. Collectively, these enzymes are capable of degrading more than 20 ECM proteins, including collagen types I, III, IV, and VII, fibronectin, and elastin in addition to fibrillin (Chakraborti et al., 2003). The action of these enzymes on elastin determines the exposure of negatively charged residues which can attract calcium ions. Also proteoglycans, fibrillins and glycoproteins show specific binding sites for calcium. Therefore changes in these molecules can favour the precipitation of hydroxiapatite crystal during aging or in pathological conditions (Berenson et al., 1985; Cherchi et al., 1990; Passi et al., 1997).

6. Foetal Bovine Serum *vs* human Platelet Lysate

All cells in culture are dependent for their growth on a variety of cytokines and adhesive molecules. Foetal bovine serum (FBS) is the most widely used serum supplement for *in vitro* culture of eukaryotic cells (Hodgson et al., 1995; Jochems et al., 2002). Generally, FBS is produced from the blood drawn from a bovine fetus that is obtained from pregnant cows (Tekkatte et al., 2011). FBS composition is complex and includes low and high molecular weight proteins and lipids, and other nutrients required for cell growth. FBS can be produced in relatively large quantities, and large batches of pretested serum can be generated and distributed on a commercial basis. Because of its relatively easy production and rich content of growth factors, FBS became the “most universally applicable cell culture additive for the stimulation of cell proliferation and biological production” (Jayme et al., 1988), but it use can have some limitations (Table 2).

Table 2. Advantages and disadvantages of FBS and hPL (Hemeda et al., 2014).

FBS	hPL
Advantages:	
Broadly applicable for many different cell types	Applicable for a wide range of different cell types. Thus far, it is particularly used for human MSCs, endothelial cells and fibroblasts.
Rich in growth factors	Enriched in growth factors of the platelet fraction (such as platelet-derived growth factor).
–	Proliferation of MSCs—particularly of MSCs derived from adipose tissue—is significantly faster than in FBS.
FBS is abundantly available (as a by-product during slaughters of pregnant cows)	hPL is easily generated by freeze-thaw procedures (waste product after expiration date of platelet units).
Commercially available (high lot-to-lot variation necessitates pretesting, facilitating more profit-yielding commercial advertisement).	
Clinical trials have been performed with relatively few side effects. However, human alternatives are preferable.	–
–	Has been used in clinical trials—no critical side effects were reported.
–	No risk of xenogeneic immune reactions or transmission of bovine pathogens.
–	Can be used in autologous settings to reduce risks of contamination or immune reactions.
Disadvantages:	
The ingredients are not precisely defined.	Not precisely defined; yet, platelet units might be more standardized than bovine fetal blood.
High lot-to-lot variation (even in pooled batches).	Variation exists between individual hPLs, which can be reduced by pooling.
–	Thus far, hPL is rarely distributed commercially.
Can evoke severe immunological reactions against xenogenic serum antigens.	Immunological reactions are possible in allogeneic settings.
High endotoxin content	
Potential source of microbial contaminants, such as fungi, bacteria, viruses or prions.	–
	Danger of transmission of human diseases by known or unknown viruses such as human immunodeficiency virus and human T-lymphotropic virus (quarantine storage cannot completely exclude this risk).
	Contamination with mycoplasma should be excluded.
Animal welfare concerns during the bleeding procedure of bovine fetuses.	–

For these reasons, over the past 15 years, various human alternatives have been tested for their ability to sustain proliferation and differentiation of cells in culture, including plasma, serum, platelet concentrates and human plateley lysate (hPL).

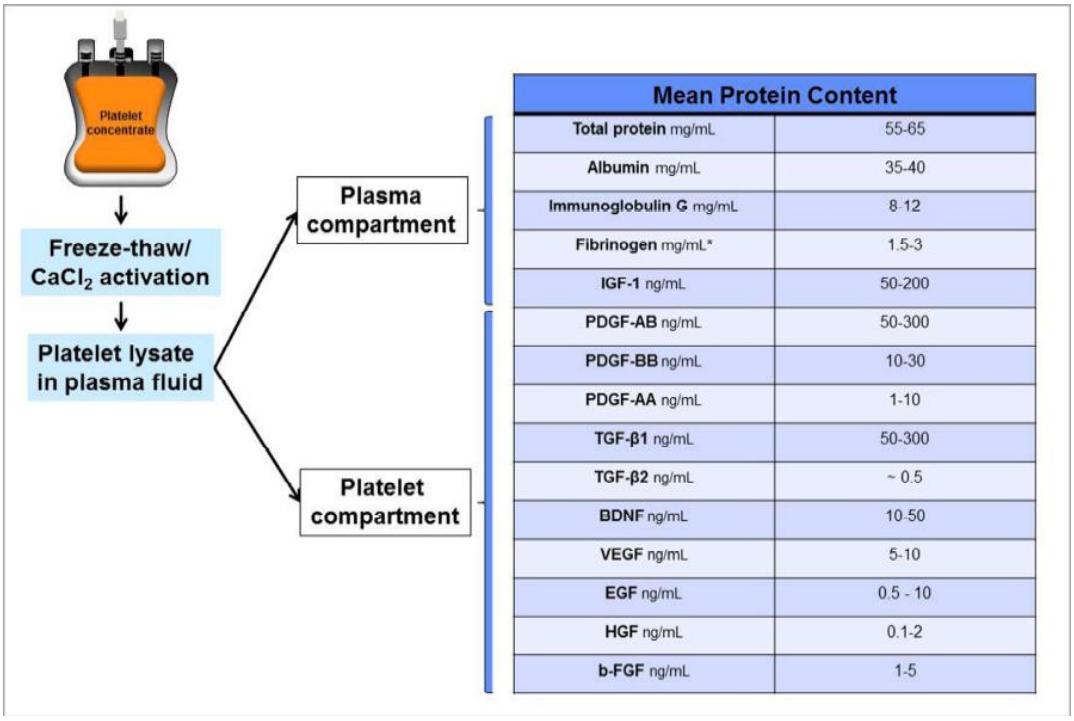
Since 1980s, many studies claim that hPL has several advantages in comparison to FBS (table 2) (Bieback et al., 2009; Müller et al., 2006; Horn et al., 2010; Lange et al., 2007) and that it can be used for the isolation and proliferation of a broad range of different cell types such as human endothelial cells (Denecke et al., 2013; Kilian et al., 2004), fibroblasts (Mirabet et al., 2008), mesenchymal stem cells from various tissues (Doucet et al., 2005 ; Schallmoser et al., 2007; Lange et al., 2007 ; Bernardo et al., 2007), periodontal ligament cells (Burnouf et al., 2012), meniscal fibrochondrocytes (Gonzales et al., 2013), chondrocytes (Kaps et al., 2002), osteocytes, myocytes, tenocytes (Mazzocca et al., 2012), annulus fibrosus cells (Pirvu et al., 2014), corneal endothelial cells (Chou et al., 2014), corneal epithelial cells (Anitua et al., 2014), indicating potential applicability in various fields of regenerative therapies.

hPL, is prepared by repeated freeze/thaw cycles, by sonication or by solvent/detergent treatment from fresh or expired platelet concentrates. The preparation of platelet concentrates as a source of

hPL has been recently reviewed (Shih et al., 2015) and technical details can be obtained from the Guide of the Council of Europe (European Directorate for the Quality of Medicines & HealthCare, 2010). Briefly, these concentrates can be prepared either from anti-coagulated whole blood following manual methods, such as the buffy coat method (largely used in Europe) or the PRP method (mostly used in the USA and Asia). Alternativeles a plateletpheresis technology (used worldwide, but more particularly in high-income countries) (Burnouf et al., 2015) can be applied. It can be partially automated as in the case of the Plateltex method (Mazzucco et al., 2008) or fully automated for the OrbiSac preparation (Janetzko et al., 2004).

Variations between individual platelet preparations can be reduced by pooling several platelet units (Rauch et al., 2011; Horn et al., 2010).

hPL, like FBS, is a complex protein mixture containing plasma protein and a series of potent bioactive mediators, primarily in their α-granules (Blair et al., 2009). Several studies have, in fact, reported that hPL contains various chemokines (Semple et al., 2011) and growth factors, such as platelet derived growth factor isoforms (PDGF-AA, -AB and -BB), TGF-β, insulin-like growth factor-1 (IGF-1), brain derived neurotrophic factor (BDNF),VEGF , epidermal growth factor (EGF), basic fibroblast growth factor (bFGF or FGF-2), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF) and BMP-2, -4, -6 (Golebiewska et al., 2015; Nurden et al., 2008) (Figure 7).



Mean Protein Content	
Total protein mg/mL	55-65
Albumin mg/mL	35-40
Immunoglobulin G mg/mL	8-12
Fibrinogen mg/mL*	1.5-3
IGF-1 ng/mL	50-200
PDGF-AB ng/mL	50-300
PDGF-BB ng/mL	10-30
PDGF-AA ng/mL	1-10
TGF-β1 ng/mL	50-300
TGF-β2 ng/mL	~ 0.5
BDNF ng/mL	10-50
VEGF ng/mL	5-10
EGF ng/mL	0.5 - 10
HGF ng/mL	0.1-2
b-FGF ng/mL	1-5

Fig. 7. Typical protein and growth factor content of human platelet lysate (HPL) prepared from a platelet concentrate obtained stabilized in plasma. * The fibrinogen content in HPL prepared by calcium chloride (CaCl2 activation) is <0.1 mg/mL) (Burnouf et al., 2015).

6.1 Platelet lysate support osteogenic differentiation

In vitro mineralization is usually obtained culturing confluent cells in the presence of high concentrations of phosphate (that can easily precipitate as soon as it forms complexes with calcium) or phosphate donor substrates (that require an active involvement of cells for the enzymatic release of inorganic phosphate from substrates). In 2006 Buranasinsup *et al* demonstrated that human dermal fibroblasts can acquire an osteoblast-like behaviour activating the mineralization process if cultured in a pro-osteogenic medium (supplemented with ascorbic acid, β-glycerophosphate and dexamethasone).

This type of medium is also able to counteract the inhibitory effects of certain proteins, such as fetuin A, present in the FBS.

Recently, the use of hPL as a culture supplement for MSC expansion has been suggested as a promising FBS substitute (Doucet et al., 2005). The expansion-promoting effect is likely due to the high concentration of natural growth factors contained in hPL, but the role of hPL in osteogenic differentiation is still under investigation (Kilian et al., 2004; Gruber et al., 2004; Graziani et al., 2006). In particular, Chevallier and coworkers (2010), studied MSC differentiation without addition of exogenous osteogenic inducers to the culture medium. A significant up-regulation of several late osteoblastic genes, *i.e*. ALP, BSP, OP, and to a lesser extent, OC, in cells cultured with the hPL was observed. In addition to the up-regulation of late osteoblastic genes, hPL supplemented medium also stimulated the expression of BMP2, a strong osteo-inductive agent (Celil et al., 2005; Matsubara et al., 2008). On the contrary, in the presence of FBS, the up-regulation of osteoblastic gene expression was only detected after the addition of exogenous inducers.

However, up-regulation of osteoblastic genes in hPL supplemented cultures, was not associated with *in vitro* mineralization. Mineralization was restored when minimal osteoblastic agents (ascorbic acid and β-glycerophosphate), were added to the culture medium (Peter et al., 1998).

Another study in 2013 demonstrated that culturing MSCs in the presence of hPL the expression of the early osteogenic marker RUNX2 was significantly higher in hPL- compared to FBS-supplemented cultures (Jonsdottir-Buch et al., 2013). RUNX2 plays a central role in committing MSCs towards the osteoprogenitor lineage and subsequently upregulates expression of other factors necessary for osteogenesis such as Wnts (Eriksen et al., 2010). High RUNX2 expression is indicative of good quality of osteogenic differentiation and limits the differentiation towards other lineages. The effect on RUNX2 gene expression is not entirely surprising, since platelet lysates can contain factors that participate in osteogenesis and normal bone turn-over, such as calcitonin and ostecalcin (Jonsdottir-Buch et al., 2013).

Furthermore, hPL's impact on MSC osteoblastic differentiation can be supported by hPL's numerous growth factors, which include BMP-2-4-6, TGF-β1, IGF, βFGF, PDEGF, PF-4, interleukin-1, and osteonectin (a major protein in mineralized bone), some of which are known to have osteo-inductive effects (van den Dolder et al., 2006; Marx et al., 1998; Koch et al., 2005; Sipe et al., 2004).

The osteogenic capacity of MSC cultured in either hPL or FBS was also investigated *in vivo* using ectopic implantation into immunodeficient SCID mice. Moreover, it has been shown that bone-forming capacity after *in vivo* ectopic implantation, was greater for MSCs pre-induced in hPL medium than in standard FBS medium (Chevallier et al., 2010).

Concluding, the use of hPL supplemented media may be advantageous for faster cell expansion and improved osteogenic differentiation as compared to the traditional use of FBS, at least in MSC.

7. AGING MODEL

During ageing, ectopic calcifications can be observed in many age-related disorders affecting soft connective tissues. In particular, a number of studies on vascular smooth muscle cells demonstrated that vascular calcification is associated to cellular senescence. Although frequently observed in the vascular system, mineral deposition may occur also in valves, cartilage, tendons, skeletal muscle and skin. However, only few data are available on other mesenchymal cells as and on their role in the age-related susceptibility of connective tissues to mineral deposition.

To understand the influence of donor's age and of replicative senescence in ectopic calcification, we have cultured neonatal (nHDF) and adult human dermal fibroblasts (aHDF) (*ex vivo* aging model) at different cumulative population doublings (CPD) (*in vitro* aging model) in a pro-calcifying environment in the presence of FBS (***FBS aging model***) (Boraldi F, Bartolomeo A, Di Bari C, Cocconi A, Quaglino D. *Experimental Gerontology* 2015).

Because it is known that biomineralization of soft connective tissue is variously influenced by mesenchymal cells type, donor's age, cumulative population doublings and presence of FBS (fetal bovine serum) or hPL (humn platelet lysate) in *in vitro* culture systems, and because there are evidence demonstrating that hPL promotes osteogenic differentiation, we have developed a platform of *ex vivo* biomineralization model, to analyze all these influencing factors. In particular we have evaluated the ability to form a calcified matrix in cultured nHDF, aHDF and hBM-MSC at low and high CPD (*in vitro* aging model) in the presence of FBS or of different commercial hPL, in a pro-calcifying medium (***hPL aging model***) (*Ex vivo* biomineralization using commercial platelet lysate is variously influenced by source, mesenchymal cell type and cumulative population doublings, *Manuscript in preparation*).

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Donor's age and replicative senescence favour the *in-vitro* mineralization potential of human fibroblasts

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ABSTRACT

Aberrant mineralization of soft connective tissues (ectopic calcification) may occur as a frequent age-related complication. Still, it remains unclear the role of mesenchymal cell donor's age and of replicative senescence on ectopic calcification. Therefore, the ability of cells to deposit *in-vitro* hydroxyapatite crystals and the expression of progressive ankylosis protein homolog (ANKH), ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), tissue non specific alkaline phosphatase (TNAP) and osteopontin (OPN) have been evaluated in human dermal fibroblasts derived from neonatal (nHDF) and adult (aHDF) donors (*ex-vivo* ageing model) or at low and high cumulative population doublings (CPD) up to replicative senescence (*in-vitro* ageing model). This study demonstrates that: 1) replicative senescence favours hydroxyapatite formation in cultured fibroblasts; 2) donor's age acts as a major modulator of the mineralizing potential of HDF, since nHDF are less prone than aHDF to induce calcification; 3) donor's age and replicative senescence play in concert synergistically increasing the calcification process; 4) the ANKH + ENPP1/TNAP ratio, being crucial for pyrophosphate/inorganic phosphate balance, is greatly influenced by donor's age, as well as by replicative senescence, and regulates mineral deposition; 5) OPN is only modulated by replicative senescence.

1. Introduction

Ageing connective tissues undergo progressive morphological, biochemical and functional modifications contributing to fibrosis and/or to loss of elasticity, thus reducing the ability to respond to mechanical stress and increasing the risk of age-related diseases. Within this context, vascular ageing and a number of pathologic conditions (*i.e.* diabetes, end-stage renal disease, inflammation and oxidative stress) are characterized by ectopic calcification that is considered a strong predictor of cardiovascular events (Giachelli et al., 2005; Shao et al., 2010). Although frequently observed in the vascular system and described since the 19th century (Burger, 1947), mineral deposition may occur also in valves, cartilage, tendons, skeletal muscle and skin (Selye, 1962; Kirsch, 2012; Siddiqui and Altorok, 2015; Endo, 2015). However, it has to be mentioned that ectopic calcification, even within a tissue, is not a diffuse process, but it is limited to specific areas, consistent with the complexity of cellular and/or extracellular factors locally acting as promoters or inhibitors of hydroxyapatite deposition. Moreover, mesenchymal cells, producing and secreting the majority of extracellular components, modulate connective tissue homeostasis, playing a key role in several pathologic conditions and in age-related complications as those due to aberrant mineralization (Ronchetti et al., 2013). Despite the life-threatening consequences of ectopic calcification, most studies have mainly investigated vascular smooth muscle cells (VSMC) and only few data are available on other mesenchymal cells and on their role in the age-related susceptibility of connective tissues to mineral deposition (Ronchetti et al., 2013). It has been reported that vascular calcification is associated to cellular senescence suggesting that this behaviour is limited to VSMC (Burton et al., 2010). The observation that altered phosphate regulation can be responsible for the mineralization of soft connective tissues in premature ageing syndromes (Mackenzie and MacRae, 2011; Shanahan, 2013) further sustains the link between ageing and ectopic calcification and underlines the importance of the phosphate circuitry in these events.

Aim of the present study was to investigate whether human dermal fibroblast cell lines, derived either from neonatal or adult donors and cultured upon senescence, have a different ability to form a calcified matrix and to exhibit changes in the expression of proteins, that, being related to inorganic pyrophosphate/phosphate (PPi/Pi) balance, have been demonstrated to regulate vascular calcification (Giachelli et al., 2005). In particular, Pi acts as a signalling molecule and a necessary source for hydroxyapatite formation, whereas PPi acts as a potent inhibitor, but it can be quickly and efficiently hydrolysed by tissue-non specific alkaline phosphatase (TNAP), releasing Pi and loosing its inhibitor activity. The levels of PPi and Pi are therefore tightly regulated by TNAP, but also by other molecules such as progressive ankylosis protein homolog (ANKH) and pyrophosphatase/phosphodiesterase 1 (ENPP1) (Rodrigues et al.,

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0531-5565/

2012). The first is a multi-pass trans-membrane protein mediating intracellular to extracellular channelling of PPi (Hakim et al., 1984; Ho et al., 2000), whereas the latter generates PPi from nucleoside triphosphates (Terkeltaub et al., 1994; Johnson et al., 1999). Therefore, changes in PPi/Pi ratio can induce or inhibit calcium-phosphate salt deposition. In addition, phosphate availability can also modulate osteopontin (OPN) expression, an inhibitor of hydroxyapatite crystal growth.

We have already demonstrated that human dermal fibroblasts from donors of different age (Boraldi et al., 2003) or cultured *in-vitro* for several passages upon senescence represent useful and informative models to investigate age-related phenotypic changes (Boraldi et al., 2010). Therefore, we have used both the *ex-vivo* and *in-vitro* ageing models to better understand whether fibroblasts cultured in standard or in calcifying media may exhibit an age-dependent increased susceptibility to pro-mineralizing factors, especially focusing on those related to the phosphate circuitry.

2. Methods

2.1. Cell culture

Neonatal (nHDF) (Cat # C-004-5C) and adult human dermal fibroblasts (aHDF) (Cat # C-013-5C) were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Cells were grown in DMEM supplemented with 10% foetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific) according to standard procedures (Quaglino et al., 2000). Initially, cell lines were sub-cultured weekly, whereas slow growing cultures were sub-cultured biweekly. The number of population doublings (PD) was calculated using the formula (van der Loo et al., 1998):

$$PD = \ln(\text{number of cells harvested}) - \ln(\text{number of cells seeded}) / \ln 2.$$

The calculated population doubling increase was then added to the previous population doubling value, to yield the cumulative population doubling level (CPD). The end of the replicative lifespan was defined by failure of cell population to double after 20 days in culture despite 3 changes/week of the culture medium.

In this study, we have analysed cell lines at low CPD (*i.e.* CPD values between 4 and 6 in both nHDF and aHDF) and at high CPD (*i.e.* CPD values between 61 and 64 and 45–49 for nHDF and aHDF, respectively).

Cells were always grown in parallel and regularly observed under the inverted light microscope.

2.2. β-galactosidase (β-gal) activity

The fluorogenic substrate C_{12}FDG (ImaGene Green) (Thermo Fisher Scientific) was used in the presence or absence of chloroquine, an inhibitor of endogenous β-gal. C_{12}FDG is a membrane permeable non-fluorescent substrate for β-gal, which, after hydrolysis of the galactosyl residues, emits a green fluorescence that remains confined within the cell. Fibroblasts were plated in 35 mm dishes at the density of 1.2×10^5 cells. After 2 days from seeding, the majority of cells were still in a proliferative state. Therefore, some dishes were pre-treated with 300 mM chloroquine for 90 min at 37 °C. All dishes were then incubated with 300 mM of C_{12}FDG for 1 h. Cells were washed in ice-cold PBS, detached with trypsin and centrifuged. The obtained pellets were re-suspended in 300 μl of PBS and ten thousand events were analysed. Fluorescence was measured at the emission wavelength of 520 nm. Experiments were conducted three times in duplicate.

2.3. In-vitro mineralization assay

Fibroblasts were routinely grown in standard medium supplemented with 10% FBS up to confluence (DMEM). For mineralization experiments, confluent cells were maintained in culture with calcifying medium (CM) comprised of DMEM supplemented with ascorbic acid (50 μg/ml) (Sigma, St. Louis, MO); β-glycerol phosphate (10 mM) (Sigma) and dexamethasone (10 nM) (Sigma) (Boraldi et al., 2013, 2014). After 10–20–30 days, mineralization was assessed by phosphate staining in the extracellular matrix with von Kossa method. Areas of mineralization were quantified on digital images by Image software. Experiments were conducted three times in triplicate.

2.4. Tissue-non specific alkaline phosphatase (TNAP) activity

TNAP activity was measured spectrophotometrically at 405 nm on nHDF and aHDF at both low and high CPD cultured in DMEM or in CM and values of optical density were related to 1×10^6 cells. Experiments were performed three times in triplicate.

2.5. Western blot

Cells were washed several times with phosphate-buffered saline and homogenized in RIPA buffer (50 mM Tris, pH 7.5, 0.1% Nonidet P-40, 0.1% deoxycholate, 150 mM NaCl, and 4 mM EDTA) (Sigma) in the presence of protease inhibitors (Sigma). Cellular lysates were centrifuged at 15,000 rpm for 20 min to clear cell debris, and supernatants were collected and stored at −80 °C until analysis. Protein concentration in the cellular extracts was determined using the Bradford method (Bradford, 1976). Protein extracts (40 μg proteins/lane) for each cell line and for each culture condition were separated by electrophoresis on 10-lane 10% polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked in TBS + 0.1% Tween 20 (TBST) + 5% non-fat dry milk for 1 h at room temperature. Primary antibodies were diluted in TBST +2.5% non-fat dry milk as follows: (i) progressive ankylosis protein homolog (ANKH) 1:1000 (rabbit polyclonal, Santa Cruz Biotechnology, Dallas, TX); (ii) ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) 1:500 (rabbit polyclonal, Santa Cruz Biotechnology); (iii) osteopontin (OPN) 1:600 (rabbit polyclonal, Santa Cruz Biotechnology). Membranes were incubated with primary antibodies overnight. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, UK, diluted 1:5000) were used after 3 washes of membranes in TBST. Western blots were visualized using Super Signal West Pico (Pierce-Thermo Fisher Scientific) according to manufacturer's protocols. Experiments were performed two times in triplicate for each cell line and culture condition.

2.6. Data analysis

Statistical analysis was performed using GraphPad Prism software, version 5.01 for MAC (GraphPad Software, San Diego, CA, USA). Data sets were compared by ANOVA or by non-parametric analysis of Mann–Whitney test and p values less than 0.05 were considered statistically significant.

3. Results

3.1. Morphology, cell proliferation and β-gal activity

Independently from donor's age, both neonatal and adult fibroblasts at low CPD exhibited the elongated shape typical of mesenchymal cells (Fig. 1A and B), but nHDF reached confluence in a shorter time compared to aHDF (data not shown). At high CPD, when cells were close to replicative senescence, all fibroblasts appeared larger with abundant vacuolated cytoplasm leaving numerous empty spaces on the plate surface even after several days of culture (Fig. 1C and D). Replicative senescence was confirmed by increased

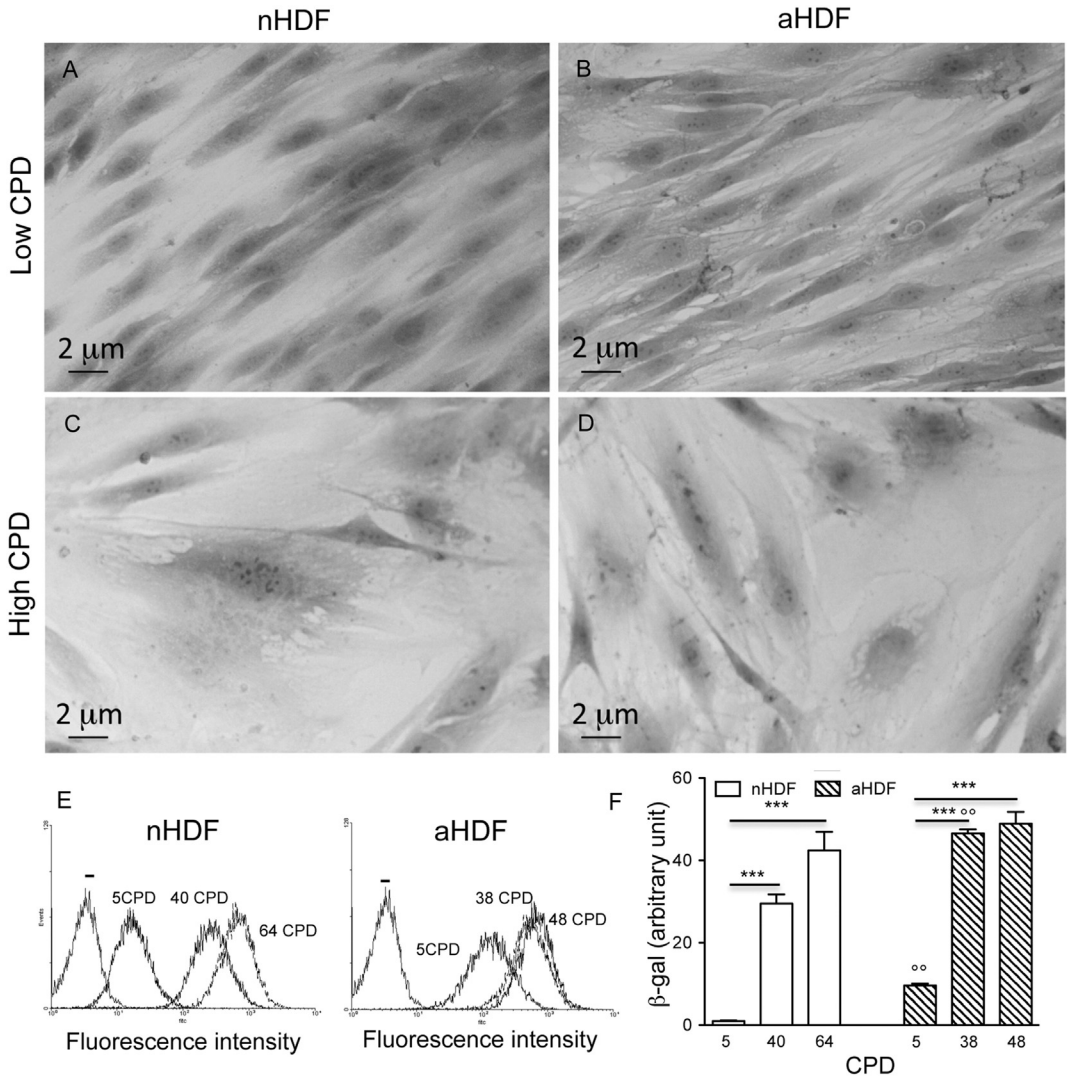


Fig. 1. Cell morphology by light microscopy (A–D) and β-galactosidase activity (β-gal) by flow cytometry (E–F). Evaluations were performed in neonatal (nHDF) and adult human (aHDF) dermal fibroblasts, at both low and high CPD, cultured in standard medium. When cells reached replicative senescence, they exhibited an enlarged and irregular shape as well as several vacuoles (C,D). A representative experiment of β-gal measurement is reported in panel E. Cells pre-treated with chloroquine (−) were used as a negative control, thus indicating that enzyme activity was only of intracellular origin. The number of positive cells is revealed by the shift of fluorescence intensity values on the abscissa. The effects of *ex-vivo* ageing (nHDF *vs* aHDF) and *in-vitro* ageing (different CPD within the same cell line) on β-gal are shown in panel F. Data are expressed as mean values ± SEM and compared with those obtained in nHDF at 5 CPD that was set at 1. ***$p < 0.0001$ 5 CPD *vs* other CPD in the same cell line; °°$p < 0.001$ nHDF *vs* aHDF at comparable CPD.

β-gal activity. The number of β-gal positive cells, as revealed by the shift of fluorescence intensity values, rose progressively with increased CPD (Fig. 1E and F). To be noted that the lifespan of cultured nHDF and aHDF, represented by the maximum number of CPD obtained *in-vitro*, was lower in aHDF (48 CPD) compared to nHDF (64 CPD) ($p \leq 0.05$).

3.2. Mineral deposition in ex-vivo and in in-vitro ageing models

Neonatal and adult fibroblasts, at both low and high CPD, were cultured in parallel in standard and in pro-calcifying media.

As expected, in standard medium all fibroblasts never exhibited mineral deposition (Fig. 2).

Calcification started to be clearly visible around 20 days of culture in pro-calcifying medium, however the extent of mineralization was highly variable depending on both donor's age and number of CPD. In particular, few small precipitates were observed in nHDF at low CPD, whereas their number and size progressively and significantly increased at higher CPD (Fig. 3A). At 30 days of culture in CM, the area covered by mineral deposits on the cellular monolayer was more than 20% and less than 4% at high and low CPD, respectively (Fig. 3B). A similar trend was observed also in aHDF (Fig. 4A), however the amount of mineral deposits was markedly increased at all times compared to nHDF (Figs. 3 and 4). At 30 days of culture in CM, the mineralized area measured in aHDF at low CPD was approximately 20% of the cellular monolayer (Fig. 4B), whereas adult fibroblasts at high CPD were almost completely mineralized (99%) (Fig. 4B).

3.3. TNAP activity

Since mineral deposition is, at least in part, due to TNAP, the enzyme activity was measured in all experimental conditions (Fig. 5). TNAP activity significantly increased over time in cells cultured in CM and values were higher compared to fibroblasts grown in standard medium. Although there was not a direct correlation between enzyme activity and the extent of mineralization, nevertheless, in the same cell line, values were significantly increased in fibroblasts at high compared to

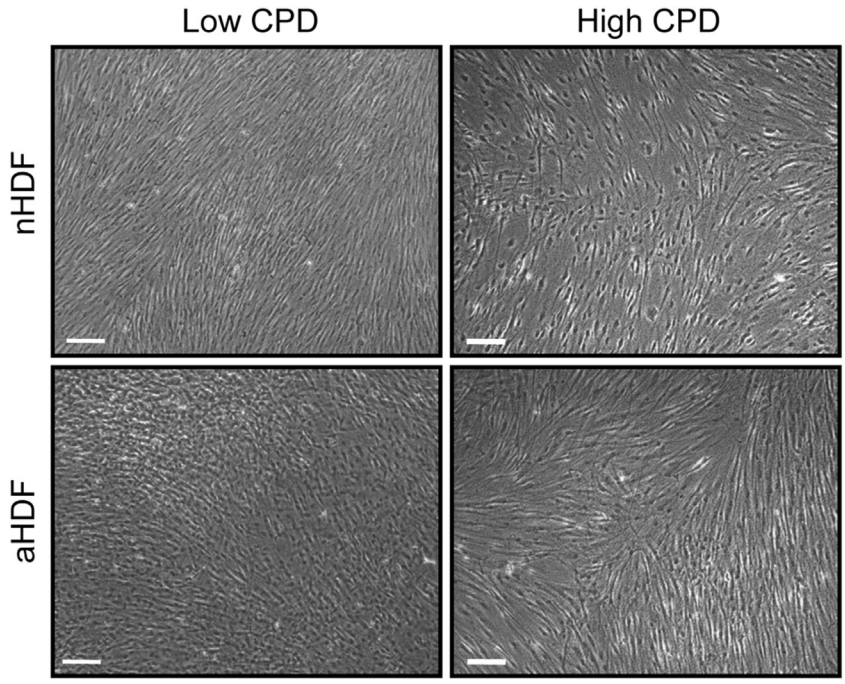


Fig. 2. von Kossa staining in standard conditions. Neonatal (nHDF) and adult (aHDF) human dermal fibroblasts, at both low and high CPD, were cultured in DMEM. Absence of mineral deposition was evident after von Kossa staining. Bar: 120 μm.

low CPD (Fig. 5A and B). Moreover, starting from the 20th day of culture in CM, TNAP activity, at low CPD, was significantly higher in aHDF than in nHDF ($p < 0.05$), whereas at high CPD differences were visible already from the 10th day of culture in CM ($p < 0.01$).

3.4. Proteins expression by Western blot

Increased TNAP activity is necessary, but non sufficient, for mineral deposition. Therefore, ANKH, ENPP1 and OPN expression have been

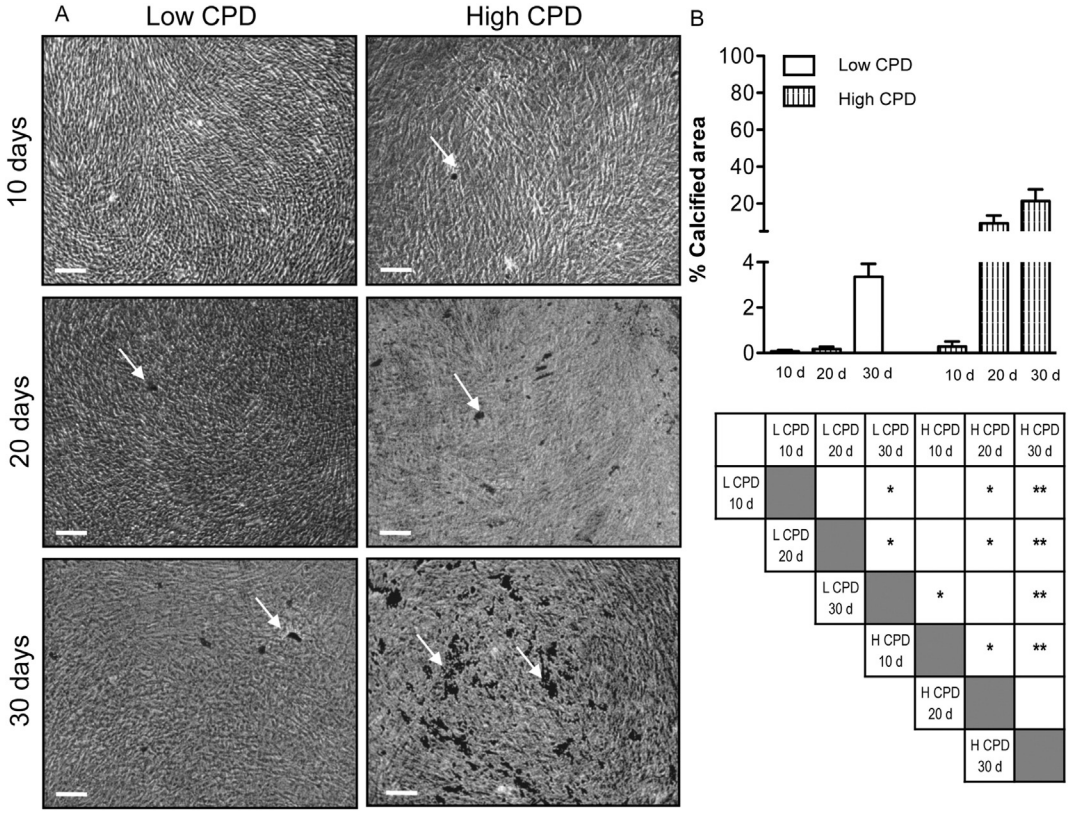


Fig. 3. *In-vitro* calcification assay. (A) Neonatal human dermal fibroblasts (nHDF), at both low (L) and high (H) CPD, were cultured in calcifying medium for 10–20-30 days (d). Hydroxyapatite deposition (arrows) was assessed by phosphate staining in the extracellular matrix with the von Kossa method. Bar: 120 μm. (B) Histogram quantifies the amount of calcified areas at different culture time points. Data are expressed as mean values ± SEM. *$p < 0.05$; **$p < 0.01$.

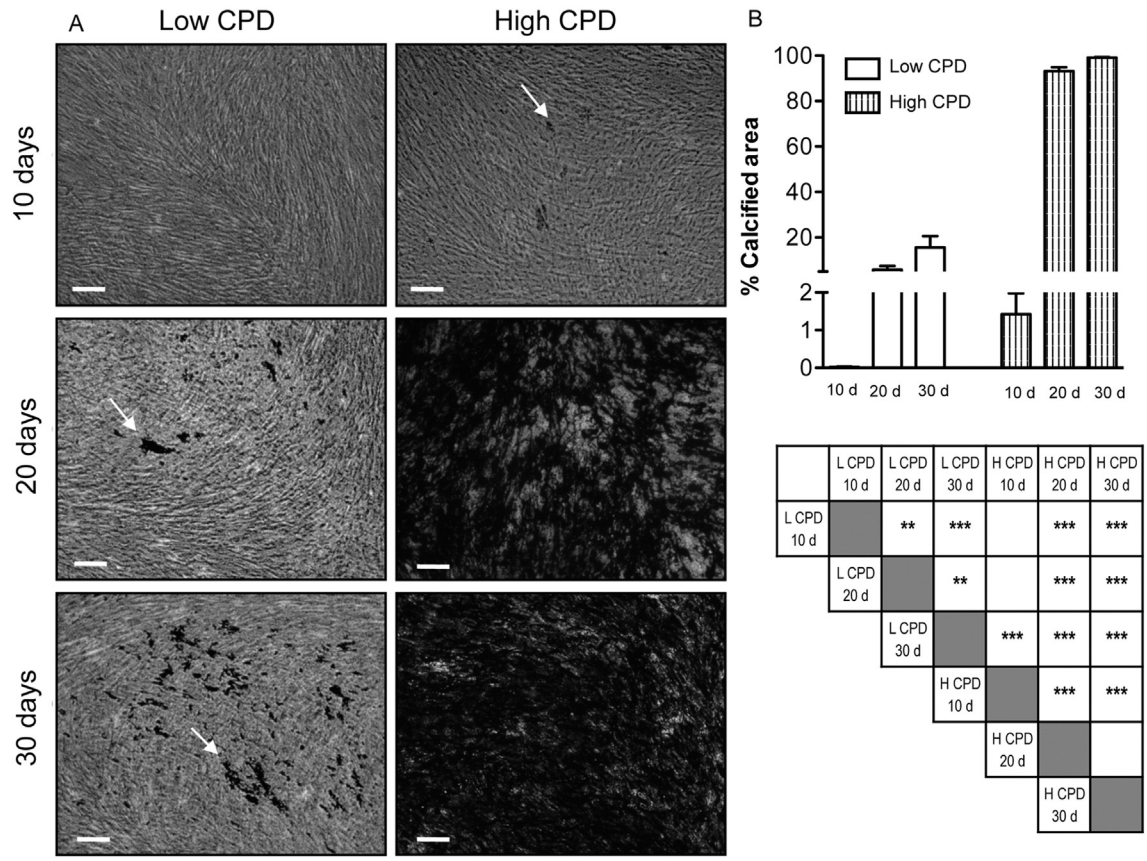


Fig. 4. *In-vitro* calcification assay. A) Adult human dermal fibroblasts (aHDF), at both low (L) and high (H) CPD, were cultured in calcifying medium for 10–20–30 days (d). Hydroxyapatite deposition (arrows) was assessed by phosphate staining in the extracellular matrix with the von Kossa method. Bar: 120 μm. (B) Histogram quantifies the amount of calcified areas at different culture time points. Data are expressed as mean values ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

quantified by Western blot, since the first two proteins are directly involved in PPi/Pi balance, and OPN is regulated by changes in Pi availability (Fig. 6).

In order to assess fibroblasts' response to pro-calcifying factors and the influence of ageing *per se*, protein expression has been evaluated in CM and in DMEM, respectively.

The amount of ANKH in nHDF was not modified by *in-vitro* senescence, neither by the presence of calcifying factors. By contrast, in aHDF, the expression of ANKH was influenced by *in-vitro* senescence, since values increased in cells cultured in DMEM at high CPD. Moreover, at low CPD, ANKH expression in standard medium was significantly reduced in aHDF compared to nHDF, indicating an effect of donor's age. When aHDF were cultured in a mineralizing environment, ANKH expression increased only at high CPD.

When cells were cultured in DMEM, ENPP1 expression was significantly increased at high compared to low CPD in nHDF. By contrast, in CM, ENPP1 was up-regulated at low CPD, whereas it was unchanged at higher CPD. Interestingly, the very low levels of protein expression observed in aHDF, both at low and high CPD in DMEM, appeared to be progressively up-regulated in CM.

In DMEM, OPN was similarly expressed in both nHDF and aHDF at low CPD and was up-regulated by replicative senescence. OPN expression remained unmodified in the presence of CM.

3.5. Proportional contribution of proteins regulating PPi levels

ANKH, ENPP1 and TNAP play in concert regulating intra-and extracellular PPi levels. In order to better evaluate the occurrence of a protein expression shift towards a pro-mineralizing condition, it is important to look at the proportional contribution of each protein. The 100% stacked column chart has been applied to further highlight the significance of results. In particular, we have compared the percentage of each protein expression in two representative experimental conditions [*i.e.* a condition never associated to mineralization (DMEM) and a condition associated to a variable amount of calcification (30 days in CM)] (Fig. 7). If the ANKH + ENPP1/TNAP ratio in nHDF at low CPD cultured in DMEM is normalized to 100%, values decreased to 87% and 75% in nHDF in CM at low and high CPD, respectively, and to 21% and 15% in aHDF in CM at low and high CPD, respectively.

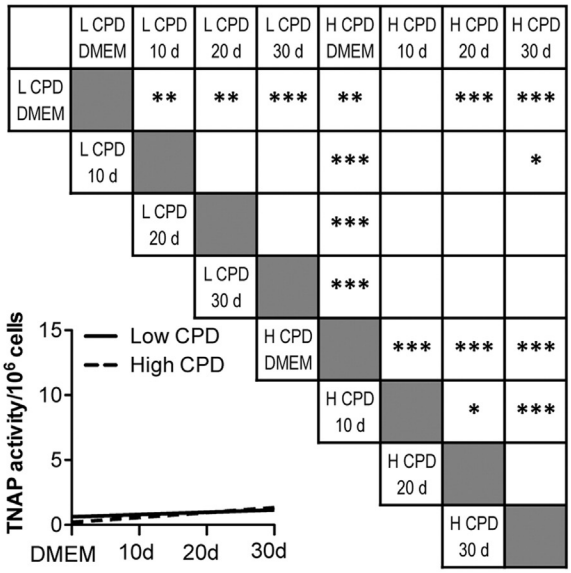
4. Discussion

Pathologic calcification of soft connective tissues represents a complication contributing to age-related tissue dysfunction, nevertheless only few studies have investigated whether the ageing phenotype of mesenchymal cells is associated or not with an increased response to pro-calcifying stimuli (Mackenzie and MacRae, 2011). Data obtained so far, focusing on vascular smooth muscle cells, lead to the hypothesis that the increased susceptibility to mineralizing stimuli of senescent cells may be peculiar of this cell type (Burton et al., 2010).

Therefore, we have compared the behaviour of two fibroblast cell lines (*ex-vivo* ageing model) derived from neonatal and from adult individuals, the first being minimally influenced by exogenous and/or by the extracellular environment, the latter having experienced several stresses contributing to epigenetic modifications and to age-related changes (Aviv, 2004). Nevertheless, when cells are placed in culture, independently from the donor's age, they may undergo a phenotypic selection. Therefore, as we have already demonstrated, fibroblasts cultured *in-vitro* for several passages, upon reaching replicative senescence, may represent a condition (*in-vitro* ageing model) mimicking more closely changes occurring during *in-vivo* ageing, at least for a number of parameters (Boraldi et al., 2010).

A

Culture condition	Time	nHDF	
		Low CPD	High CPD
DMEM		0.523±0.08	0.122±0.01
CM	10 d	0.947±0.06	0.685±0.02
	20 d	0.989±0.03	1.026±0.08
	30 d	1.049±0.03	1.276±0.09



B

Culture condition	Time	aHDF	
		Low CPD	High CPD
DMEM		0.044±0.01	0.077±0.01
CM	10 d	0.612±0.15	2.936±0.03
	20 d	2.666±0.05	10.952±0.02
	30 d	2.823±0.27	6.969±0.21

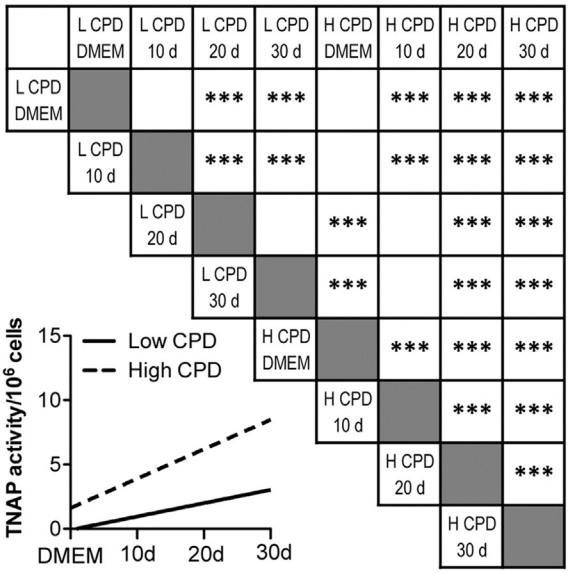


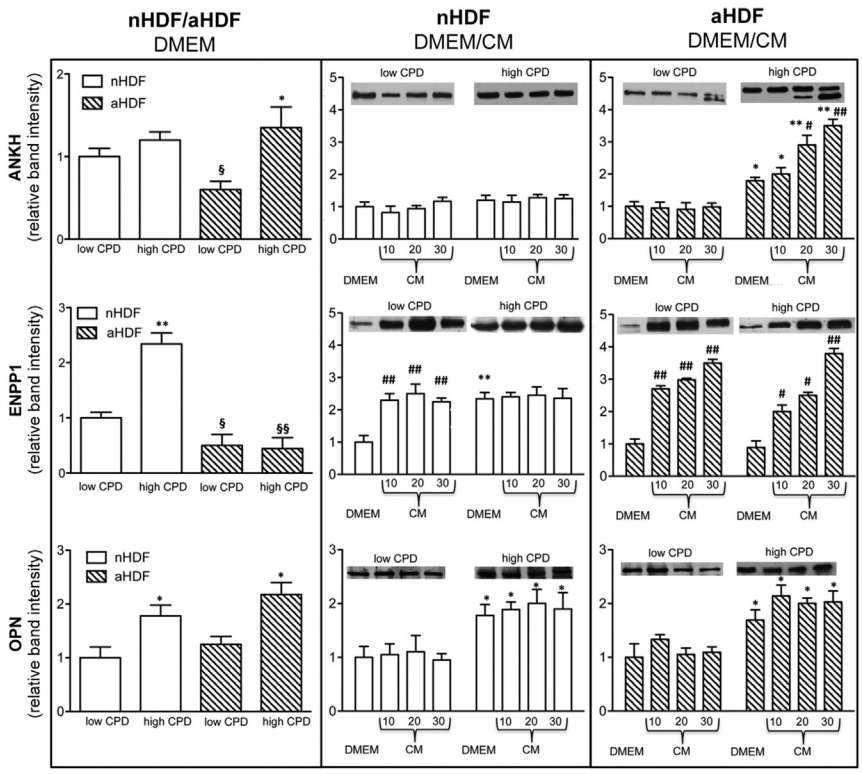
Fig. 5. TNAP activity. Enzyme activity was measured in neonatal (nHDF) (A) and adult (aHDF) (B) human dermal fibroblasts, at both low (L) and high (H) CPD. Fibroblasts were cultured until confluence with standard medium (DMEM) and then with calcifying media (CM) for 10–20–30 days (d). TNAP activity for 1×10^6 cells is expressed as mean values ± SEM. The time-dependent trend of TNAP activity in both cells lines is shown in graphs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The use of these models allows to better understand if donor's age and/or replicative senescence influence the mineralizing potential of mesenchymal cells other than vascular smooth muscle cells (*i.e.* dermal fibroblasts).

We have observed an inverse relationship between donor's age and proliferative lifespan in agreement with Martin et al. (1970), whereas other studies (Cristofalo et al., 1998; Boraldi et al., 2010) failed to find this correlation. This discrepancy may be due to the fact that in the present study we have compared fibroblasts from neonatal donors with those from adult individuals, whereas in other studies fibroblasts from adult and old donors were compared with those from young subjects. Consistently, our laboratory demonstrated that in the skin equivalent model, best results, in terms of matrix production and cell proliferation, were obtained with neonatal fibroblasts compared to young or adult cells (Croce et al., 2004). Taken together these results indicate that fibroblasts loose their juvenile behaviour quite early, even when cells are placed in optimum culture conditions. It may be suggested that in addition to intrinsic (*i.e.* genetic) factors, extrinsic influences (*i.e.* life style and exposure to environmental noxae) may trigger, for instance, genome instability, telomere attrition and loss of proteostasis acting on proliferative lifespan and on the capacity of these cells to secrete extracellular matrix proteins.

Moreover, in agreement with previous data (Kurz et al., 2000; Maier et al., 2007; Boraldi et al., 2010), nHDF and aHDF are characterized by the presence, already at low CPD, of β-gal positive cells, indicating that also *in-vitro* senescent and quiescent cells coexist. Morphological changes and increased β-gal activity observed in both cell lines were associated with reduced growth capacity and therefore with increased CPD, however the replicative lifespan was mainly influenced by donor's age.

To induce *in-vitro* calcification, cells were cultured in CM at both low and high CPD. This medium is necessary since mesenchymal cells, in standard culture conditions, are not able to promote mineralization due to the presence of serum inhibiting molecules as fetuin (Buranasinsup et al.,



ANKH					
Culture condition/Time		nHDF		aHDF	
		Low CPD	High CPD	Low CPD	High CPD
DMEM		**1,0**	1,2	0,6	1,3
CM	10 d	0.8	1,1	0,6	1,3
	20 d	0.9	1.2	0,5	1,7
	30 d	1.1	1.2	0,6	2,1

ENPP1					
Culture condition/Time		nHDF		aHDF	
		Low CPD	High CPD	Low CPD	High CPD
DMEM		**1,0**	2,3	0,5	0,4
CM	10 d	2,3	2,4	1,3	1,0
	20 d	2,5	2,4	1,5	1,2
	30 d	2,2	2,4	1,7	1,9

OPN					
Culture condition/Time		nHDF		aHDF	
		Low CPD	High CPD	Low CPD	High CPD
DMEM		**1,0**	1,8	1,2	2,1
CM	10 d	1,1	1,9	1,5	2,5
	20 d	1,1	2	1,2	2,4
	30 d	0,9	1,9	1,3	2,4

Fig. 6. ANKH, ENPP1 and OPN expression by Western blot. Neonatal (nHDF) and adult (aHDF) human dermal fibroblasts were cultured at confluence in standard medium (DMEM) and in calcifying medium (CM) for 10, 20 and 30 days (d) at both low and high CPD. In nHDF/aHDF-DMEM panels, each protein expression is compared with values of the same protein in nHDF at low CPD which has been set at 1 in order to assess age-related changes independently from the presence of pro-calcifying factors. In nHDF-DMEM/CM and aHDF-DMEM/CM panels, each protein expression is compared with values of the same protein in nHDF and aHDF at low CPD in DMEM, respectively, which has been set at 1 in order to evaluate, for each cell line, the time-dependent influence of CM on protein expression also in relation to increased CPD (*i.e.* replicative senescence). On top of each panel a representative Western blot is shown. Data are expressed as mean values ± SEM; *$p < 0.05$; **$p < 0.01$ high CPD *vs* low CPD in the same cell line; §$p < 0.05$; §§$p < 0.01$ aHDF *vs* nHDF at the same CPD; #$p < 0.05$; ##$p < 0.01$ CM *vs* DMEM. Tables at the right side of the figure show changes of protein expression in nHDF and in aHDF cultured in DMEM and in CM at low and high CPD. All experimental conditions, for each protein, are compared with value of nHDF at low CPD in DMEM which has been set at 1 in order to evaluate, as a trend, the combined influence of CM, donor's age and replicative senescence.

2006; Boraldi et al., 2013). The amount of hydroxyapatite deposition (Boraldi et al., 2014) depends on time in culture (up to 30 days in CM), donor's age (neonatal and adult) and cellular senescence (low and high CPD), in agreement with the *in-vivo* prevalent occurrence of calcification in ageing soft connective tissues.

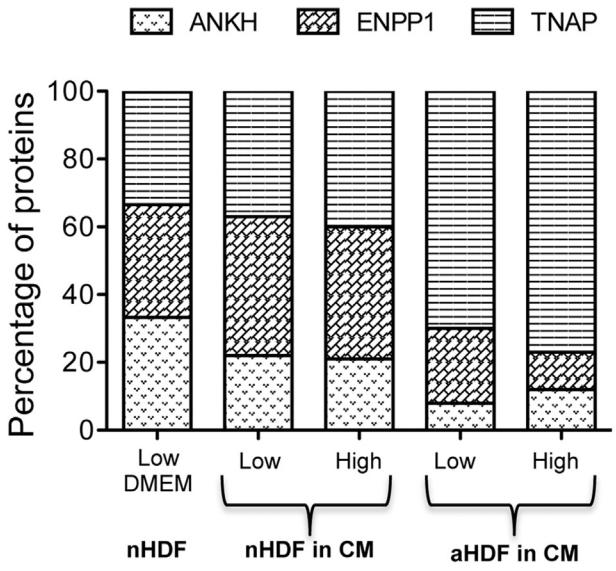


Fig. 7. The 100% stacked column charts are used to graphically visualize the percentage contribution of ANKH, ENPP1 and TNAP in neonatal (nHDF) and adult (aHDF) human dermal fibroblasts cultured in calcifying medium (CM) for 30 days at both low and high CPD in comparison with nHDF at low CPD cultured in DMEM.

Although mineral deposition is a very complex process involving numerous promoting and inhibiting factors, it has been observed in VSMC that phosphate availability plays a key role in controlling pathologic calcification (Giachelli et al., 2005).

Within this context, TNAP is an ectoenzyme capable to dephosphorylate a broad range of molecules (*i.e.* phosphoproteins, PPi, β-glycerophosphate) favouring mineral deposition and crystal growth (Millán, 2006). In both the *ex-vivo* and *in-vitro* ageing models, there was a progressive increase of TNAP activity, even if nHDF exhibited values significantly lower than aHDF. As already demonstrated by Mendes et al. (2004), there is not a direct correlation between TNAP activity and the amount of deposited mineralized matrix, since TNAP *per se* is necessary, but not sufficient to induce mineral deposition (Boraldi et al., 2013) and a threshold PPi/Pi ratio must be reached. After 30 days of culture in CM, nHDF were always less susceptible to pro-calcified factors in comparison with aHDF. Cells from neonatal donors were characterized by: high levels of ENPP1, stable ANKH expression and only a moderate increase of TNAP activity. The resistance to calcification seems to be associated to the high expression of ENPP1 triggering the formation of PPi capable to inhibit hydroxyapatite deposition. On the contrary, aHDF exhibited increased ANKH and ENPP1, although ENPP1 never reached the levels of nHDF. These changes, associated with a strong increase of TNAP activity, may explain, at least in part, the abundance of hydroxyapatite in aHDF cultures. In fact, although ENPP1 up-regulation can increase PPi production, this inhibitor is

actively hydrolysed by the huge increase of TNAP, thus releasing Pi that is responsible for the extensive mineral deposition revealed by von Kossa staining.

The concerted action of TNAP, ANKH and ENPP1 regulates PPi levels, therefore when ANKH and ENPP1 overcome TNAP (as in HDF cultured in DMEM) hydroxyapatite deposition is never observed, by contrast, when TNAP prevails on ANKH and ENPP1 (as in aHDF at high CPD in CM) there is an almost complete mineralization of the cellular monolayer. These effects are mainly dependent on donor's age and are further modified by replicative senescence.

Several reports have suggested that, in VSMC, osteopontin acts as an inhibitor of calcification, being calcium-dependent and associated with apatite crystal growth (Wada et al., 1999). Although it has been demonstrated that there is a tight link between osteopontin expression and phosphate levels (Beck et al., 2000), this protein does not interfere with TNAP activity or with phosphorus levels (Wada et al., 1999). Therefore, we have investigated the expression of this protein in *ex-vivo* and *in-vitro* ageing models and in the presence of CM in order to evaluate if the expression of osteopontin can be modulated by a medium providing phosphate-releasing substrates. We have found that replicative senescence, in both cell lines, induced increased OPN levels in agreement with previous findings in senescent fibroblasts (Pazolli et al., 2009; Liu et al., 2012), but OPN expression was never modified by the presence of CM. These data further sustain the complex role of this multifunctional matri-cellular protein that seems to act as a regulator of mineral deposition mainly in conditions of injury and disease (Giachelli et al., 2005), whereas its role as an endogenous natural inhibitor is still questionable (Lomashvili et al., 2004).

5. Conclusion

In summary, this study highlights, for the first time, that: 1) replicative senescence favours hydroxyapatite formation in fibroblasts as shown in VSMC (Burton et al., 2010), demonstrating that this behaviour is not limited to a specific mesenchymal cell type; 2) donor's age acts as a major modulator of the mineralizing potential of HDF, since nHDF are less prone than aHDF to induce calcification in all examined experimental conditions; 3) donor's age and replicative senescence play in concert synergistically increasing the calcification process; 4) the ANKH + ENPP1/TNAP ratio is crucial for mineral deposition and is dramatically influenced by donor's age but also by replicative senescence; 5) OPN is only modulated by replicative senescence (Table 1).

In the light of these results, it could be suggested that ageing human fibroblasts may have a strong mineralizing potential and this may be also the consequence of epigenetic changes that are known to accumulate throughout life (Robert et al., 2009) and that may trigger the reduced capacity of fibroblasts to inhibit the calcification process.

Conflict of interest

No conflicts of interest are declared.

Table 1
Influence of *ex-vivo* (nHDF *vs* aHDF cultured in CM at low CPD) and *in-vitro* (low CPD *vs* high CPD in the same cell line cultured in CM) ageing on calcification-related parameters.

Parameters	Ageing	
The whole table should be or centred or left aligned	*Ex-vivo*	*In-vitro*
Mineral deposition	✓	✓
TNAP activity	✓	only in aHDF
ANKH expression	✓	only in aHDF
ENPP1 expression	✓	–
ANKH + ENPP1/TNAP	✓	only in aHDF
OPN expression	–	✓

Acknowledgments

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***Ex vivo* biomineralization using commercial platelet lysate is variously influenced by source, mesenchymal cell type and cumulative population doublings.**

Abstract

Background. *Ex vivo* calcification takes place only when cells are cultured in a specific environment providing adequate supplementation of pro-osteogenic agents. In recent years, platelets are highly influential contributors to biomineralization in both physiological and pathological microenvironments and human Platelet Lysate (hPL) is increasingly favoured as an FBS replacement in cell culture medium for clinical applications in regenerative medicine. However, hPL can be obtained using different methods that may influence platelet number and growth factor concentration and consequently osteogenic differentiation. Moreover, *ex vivo* biomineralization outcome appears to be highly variable depending, beside hPL sources, also on ageing and cell type. Therefore, we compared diverse mesenchymal cell types (neonatal and adult derived dermal fibroblasts and hBM-MSC) of different extracellular matrix biomineralization potencies, and evaluated the influence of *ex vivo* (neonatal *vs* adult donors) and *in vitro* (low and high cumulative population doublings) aging on matrix mineralization.

Methods. Cells were cultured for 9, 14 and 21 days in calcifying media supplemented with FBS or with different commercial preparations of hPL. Different hPL sources were analysed by LC-ESI-Q-TOF. Ex vivo matrix mineralization was assessed by von Kossa staining.

Results. Mass spectra indicated hPL differences between commercial sources. Time-dependent *ex vivo* biomineralization highlighted variances across mesenchymal cell types. Prompt biomineralization in hBM-MSC depends on hPL source, whereas in neonatal HDF rest both on time-course and hPL source. Biomineralization promoted by adult HDF reflected Cumulative Population Doublings.

Conclusion. We have shown that it is possible to derive unifying *ex vivo* contexts with contrasting hPL-specific biomineralization phenotypes for direct comparison of physiological and pathological biomineralization. Moreover, use of commercial hPL sources can advantageously allow scalable expansion for further studies exploring the relevant underlying molecular mechanisms.

Introduction

Biomineralization is a multifactorial complex biological process whereby, in most cases, hydroxyapatite (calcium phosphate) crystals formed within matrix vesicles bud from the surface membrane of specific cells and deposit between extracellular matrix fibrils e.g. collagen [1]. Cellular enzymes hydrolyse inorganic pyrophosphate to inorganic phosphate to promote mineralization that is further regulated by non-collagenous proteins and extrinsic growth factors [2]. The process of biomineralization in human tissues is usually physiological, during the formation of bone, yet can also be pathological, occurring inappropriately in soft connective tissues. Given its central role in the osteoblastic phenotype, biomineralization, that can be detected by von Kossa (VK) staining, has been extensively characterised in *ex-vivo* expanded populations of several human mesenchymal cells induced to undergo osteogenic differentiation [3-4]. *Ex vivo* calcification takes place only when cells are cultured in a specific environment. In fact, mesenchymal cells, independently from their ability to induce calcification *in vivo,* are unable to produce a mineralized matrix in standard medium supplemented with foetal bovine serum (FBS) because in FBS calcification inhibitor factors (i.e. fetuin-A) are present in high concentration. Therefore, to obtain *ex vivo* mineralization, culture media are supplemented with pro-osteogenic agents (i.e. Pi or β-glycerophosphate) to follow a temporal cascade of maturational stages towards the osteoblast phenotype and to counteract serum inhibitory effects [5]. In recent years, it has been also reported that platelet rich plasma (PRP) plays a role in promoting the healing of hard and soft connective tissues showing positive outcomes for bone regeneration in clinical trails [6]. Platelets are highly influential contributors to biomineralization in both physiological and pathological microenvironments, with roles in determining sites for mineral nucleation [7], provision of growth factors for osteoblasts [8] and as sources of exosomal mediators [9]. Moreover, the fractured platelet derivative human Platelet Lysate (hPL) is increasingly favoured as an FBS replacement in cell culture medium for clinical applications in regenerative medicine [10]. However, platelet concentrate and hPL can be obtained using different methods that may influence platelet number and growth factor concentration and consequently osteogenic differentiation outcomes [11-13]. Moreover, hPL effectiveness can vary widely according to donor-specific variability (i.e. age,

gender), anticoagulant (i.e. heparin) and storage [12, 14], therefore commercial sources of platelet lysate sourced from pooled donors are recommended to advantageously improve consistency and lower batch variability [15-16].

Ex vivo biomineralization is also influenced by ageing and cellular senescence [17]. Ageing, for instance, is associated with an increased incidence of biomineralization-associated diseases, including osteoporotic paucity of bone mineralization as well as excess of calcification in soft connective tissues and atherosclerotic vasculature [18]. Intriguingly, it has been proposed that replicative senescence can impair bone progenitor osteogenic differentiation, including matrix mineralization [19], but can also favour the calcification of soft connective tissues increasing the susceptibility of mesenchymal cells to pro-osteogenic stimuli [17].

Therefore, *ex vivo* biomineralization outcome appears to be highly variable depending, for instance, on hPL sources, ageing, cell type. To date a comparison of the effectiveness of these factors on the calcification process has been never performed. Given the significance of platelet fractions for mineral deposition and the influence of manufacturing processes on the characteristics of the hPL preparations [20] and consequently on their biological efficiency, we chose to compare the effect of different commercial sources of platelet lysate on *ex vivo* biomineralization. Moreover, we compared diverse mesenchymal cell types (neonatal and adult derived dermal fibroblasts and hBM-MSC) of different extracellular matrix biomineralization potencies, and evaluated the influence of *ex vivo* (neonatal *vs* adult donors) and *in vitro* (low and high cumulative population doublings) aging on matrix mineralization.

Materials and Methods

Cell culture

Neonatal (nHDF) (Cat # C-004-5C) and adult human dermal fibroblasts (aHDF) (Cat # C-013-5C) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and were grown in DMEM supplemented with 10% foetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific) according to standard procedures [21]. Human bone marrow mesenchymal stromal cell (hBM-MSC) were harvested from a 42 year old male donor with informed consent according to the declaration of Helsinki with procedures approved by local ethical committee. Bone marrow derived white blood cells were seeded at an initial density of 50,000 cells/cm^2 in

300 mL complete medium in Corning CellStack™ (Sigma, St. Louis, MO) tissue culture vessels according to a two-step hBM-MSC isolation and expansion procedure and at passage 1 (p1) hBM-MSC were immunophenotypically characterised [22-23]. nHDF, aHDF and hBM-MSC were passaged serially until they reached replicative senescence at p52; p45 and p10, respectively. Since HDFs have a longer culture lifetime and a delay of replicative senescence compared to hBM-MSC, Cumulative Population Doublings (CPD) were calculated during passage to provide cells of low CPD (exponentially grown for all cell type was p3; duplication time (DT) was ≈35 hours (h) for nHDF; 40h for aHDF and 72h for hBM-MSC) and high CPD above 85% of their total culture lifespan (p40 and p35 for nHDF and aHDF, respectively, with DT of ≈ 280h; p8 for hBM-MSC with DT of ≈ 101h).

Prior to testing biomineralization potential, all type cells were grown for at least one passage in DMEM supplemented with 10% FBS.

Cells were always grown in parallel and regularly observed under the inverted light microscope.

Ex vivo biomineralization assay

Cells were routinely grown in standard medium supplemented with 10% FBS up to confluence. For mineralization experiments, confluent cells were maintained in culture with a calcifying medium (CM) comprised of DMEM supplemented with ascorbic acid (50µg/ml) (Sigma); β-glycerol phosphate (10mM) (Sigma) and dexamethasone (10nM) (Sigma) and 10% FBS [24-25] or with different human platelet lysate (hPL). In particular, we used: two liquid formula of hPLs produced by Cook Medical (Limerick, Ireland) namely hPL1 and hPL2 that require or no heparin, respectively; a liquid formula of hPL produced by Macopharma (Tourcoing, France) namely hPL3 that required heparin addition; a lyophilized formula of hPL produced by Sclavo Diagnostics International (Siena, Italy) namely hPL4 without addition of heparin.

After 9, 14 and 21 days in CM, mineralization was assessed in the extracellular matrix by phosphate staining with the von Kossa method. Briefly, cells were fixed in 4% paraformaldehyde, stained with 2.5% silver nitrate, placed under a UV lamp for 30 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for two minutes. Von Kossa-positive (black) deposits were observed after alcohol washes. Areas of

mineralization were quantified on digital images by Image software. Experiments were performed two times in triplicate.

LC/ESI-Q-TOF MS analysis

Analyses were performed on an ESI-Q-TOF Accurate-Mass spectrometer (G6520A, Agilent Technologies), controlled by MassHunter (v. B.04.00) and interfaced with a CHIP-cube to an Agilent 1200 nano-pump.

Chromatographic separation was performed on a chip (Agilent Technologies) with a 75 μm I.D., 43 mm, 300 Å C18 column, prior to a desalting step through a 40 nL trap column. The injected sample (1 μL) was loaded onto the trap column with a 4 μL/min 0.1% FA:ACN (98:2) phase flow, and after 3 minutes, the precolumn was switched in-line with the nanoflow pump (400 nL/min, phase A: water:ACN:FA 96.9:3:0.1, phase B: ACN:water:FA 94.5:5:0.1), equilibrated in 10% B. The proteins were eluted from the RP column through the following gradient: 10–90% B in 5 minutes, hold in 90% B for 5', and switched back to 10% B in 3 minutes, for a total runtime of 40 minutes, including a 10' post-run reconditioning step.

Profile MS spectra were recorded from 350 to 3200 m/z at scan rates of 1 Hz; the detector was operated at 2 GHz in extended dynamic range mode. Mass spectra were automatically recalibrated with two reference mass ions.

The spectra were displayed and processed by the software MassHunter Qualitative Analysis (B05.00, Agilent Technologies).

Mass spectra across the whole chromatogram (0-26 minutes) were averaged and, after subtracting the background (obtained by averaging the mass spectra at the end of the run), the spectrum was deconvoluted by using the maximum entropy algorithm in the range 10000-150000 Da.

Statistical analysis

Data were expressed as mean values ± SEM of all measurements and compared by ANOVA test with significance at $p < 0.05$. Statistical data were obtained using GraphPad software, version 5.0 (San Diego, CA, USA).

Results

Mass spectra indicated hPL differences between commercial sources.

The complex protein mixtures of four hPLs without gel separation and digestion were analysed by ESI-Q-TOF. A broad range of peaks with intensity less than 0.5 across the selected 10000 to 150000 m/z range and a prominent base peak of intensity greater than 1.5 between m/z ratios 66440 to 66557 (Fig 1) were observed in all samples, however, there was a great heterogeneity in the number and peak intensities between hPLs. For example, hPL3 and hPL4 exhibited a peak at 76102 and at 109559 m/z, whereas hPL1 and hPL2 did not (Fig 1).

Time-dependent *ex vivo* biomineralization across mesenchymal cell types.

Since hPL and pro-osteogenic agents present in the culture medium affects the proliferation rate of different cell lines [26-27], all cells were cultured until confluence in a standard medium containing FBS, then medium was removed and replaced with calcifying medium (CM) supplemented with either FBS or hPL. The change from FBS to hPL-containing medium was well tolerated, without cell toxicity.

The time point of 14 days after the start of CM-mediated induction highlighted the clearest differences relevant to hPL source, mesenchymal cell type and relative cumulative population doubling (Fig 2).

Heterogeneity among hPL sources was notable in hBM-MSC at low CPD, since ≈50% of the cell monolayer area was positive for von Kossa (VK) staining when using hPL3 or hPL4, but <5% positivity was seen using induction medium supplemented with FBS, hPL1 or hPL2. In contrast, VK positivity for nHDF at low CPD at this time point was either completely negative with FBS or <5% for all hPL sources. Similarly, aHDF at low CPD showed weak VK staining with hPL, whereas some positivity was observed in FBS (white arrows, Fig 2) that was not seen in nHDF.

At high CPD there were the most striking differences. None of the hBM-MSC and consistently none of the nHDF showed VK staining significantly above 5% in all experimental conditions (Figs 2 and 3). On the contrary, aHDF in hPL, regardless of source, were stained black for over 50% of the monolayer surface (Figs 2 and 3).

Prompt biomineralization in hBM-MSC reflected hPL source.

In line with expectations from previous studies [23], treatment of positive control hBM-MSC with mineralization induction medium increased VK staining over time and at 3 weeks represented over 20% of the confluent cell monolayer area regardless of whether mineralization induction medium was supplemented with FBS or hPL (Fig 3, top row). Notably, among all experiments the only example of von Kossa staining above 10% within 9 days after induction was seen using hPL3 in low CPD hBM-MSC. A further example of heterogeneity among hPL sources in these cells was that two sources, hPL1 and hPL2, induced less than 5% biomineralization at two weeks, whereas hPL3 and hPL4 supported over ten-fold more VK positivity at low CPD. Although hPL3 and hPL4 had a similar effects at 14 and 21day of culture, at 9 days only hPL3 induced a clear calcification.

Notably, at 21 days all sources of hPL led to mineralization, but the 90% VK stained area with hPL3 and hPL4 was over twice that observed for hPL1 or hPL2 regardless of whether the hBM-MSC were at low or high CPD.

Biomineralization promoted by neonatal HDF reflected time-course and hPL source.

The overall pattern of calcification evidenced by VK staining in nHDF was very different to that seen in hBM-MSC (Fig 3, middle row). Notably use of FBS or hPL4 did not result in any appreciable calcification. There was VK staining at 21 days when using the other hPL sources. Similar to observations using hBM-MSC, the use of hPL3 led to more VK staining that hPL1 or hPL2. In contrast to hBM-MSC, however, the extent of VK staining in nHDF using hPL3 was also influenced by CPD; high CPD cells had over twice the calcification area found in low CPD cells.

Biomineralization promoted by adult HDF reflected Cumulative Population Doublings.

The pattern of calcified staining in aHDF was similar for mineralization induction medium supplemented with either FBS or hPL and it was not significantly influenced by the source of hPL (Fig 3, bottom row). For aHDF at low CPD, there was very little VK staining at 9 and 14 days, with the staining evident at 21 days not exceeding 20% of the monolayer area. In contrast, for aHDF at high CPD, there was appreciable VK positivity at 14 days, from a 10% area using FBS to a ≥50% area using hPL. At 21 days, all aHDF with high CPD were strongly

stained and the calcified area was over 70% regardless of whether FBS or hPL was used in the mineralization induction medium.

Context for physiological *versus* pathological biomineralization.

Mesenchymal cells derived from tissues that physiologically mineralize, i.e. hBM-MSC at low CPD, promptly showed mineralization at 9 or 14 days in specific hPL sources and by 21 days there was VK staining for all induction contexts (Fig 3). Notably, early time point mineralization was lost in hBM-MSC at high CPD.

For HDF derived from soft connective tissues that mineralize only in pathological situations, cells at low CPD did not show any prompt mineralization. Notably, for aHDF this changed at high CPD, and aberrant mineralization was observed promptly at 14 days. Neonatal HDF were the most resistant to *ex vivo* mineralization, without any significant prompt biomineralization, although certain hPL supplemented media could induce VK staining at 21 days. Use of hPL4 provided a unifying context that met the expectation of physiological outcomes in cells with low CPD, i.e. prompt 14-day biomineralization only in hBM-MSC, but pathological outcomes in cells with high CPD, i.e. loss of prompt biomineralization in hBM-MSC and acquisition of high aberrant mineralization in adult but not neonatal HDF.

Discussion

A demographically burgeoning need for addressing the diseases of aging has led to growing interest in understanding the processes governing biomineralization in order to improve current therapeutic interventions. However experimental models addressing biomineralization have been very diverse with regard to cell types studies and relevant reagents used, making direct comparisons difficult. Thus we conducted a comparative study that aimed to highlight key factors governing biomineralization outcomes whilst hoping to establish *ex vivo* conditions suitable for exploring both physiological (hBM-MSC) and pathological (HDF) contexts.

We tested hPL as liquid concentrates with a varied requirement for addition of heparin, versus a dry lyophilized form that was rehydrated before use as a cell culture medium supplement. Heparin, a sulphated glycosaminoglycan primarily added as an anticoagulant preventing fibrinogen conversion to fibrin so as to avoid hPL gelatinization in the medium, is

not a totally benign component. It has been demonstrated that low or high doses of heparin can oppositely favour osteogenic differentiation [28-29]. Two hPL sources (hPL1 and hPL3) recommended heparin addition when supplementing culture medium, whereas this was not necessary for hPL2 or hPL4. Mineralization outcomes were not determined by whether hPL required heparin addition or not, as clearly demonstrated comparing data obtained from hPL1 and hPL2 provided by the same company.

Interestingly, comparison of four hPLs revealed a different distribution and intensity of mass spectrometry peaks, suggesting that they have a diverse composition. Therefore, future studies are necessary to characterize these products. Notably, manufacturers' provision of pooled hPL did not necessarily avoid diverse outcomes when using hPL from different sources and this likely reflected the complex nature of hPL and its susceptibility to be influenced by manufacturing processes [20].

Platelet extract lyophilisation can advantageously preserve biological activity and complements procedures for releasing growth factors from platelet α-granules such as freeze-thaw cycles, sonication or activation by thrombin/$CaCl_2$ treatment [30]. Although freeze-thawing and cryopreservation of platelet lysate introduces different growth factor release kinetics compared to use of fresh lysate, the bioactivity is sufficiently preserved for equivalent target cell responses [31] especially when carefully rehydrated with plasma [32]. We found the lyophilized source hPL4 to be a very competent supplement for prompt mineralization in hBM-MSC. Finding a diverse range of context-specific outcomes indicated that our choice of different mesenchymal cell types and cumulative population doublings provided a suitable platform for comprehensively exploring biomineralization factors in hPL. In particular, hBM-MSC have been extensively studied in cell culture for analysis of the processes concerning bone remodelling, with skeletal stem cell differentiation to osteoblasts [33], complemented by focus on mechanisms governing biomineralization *per se* [34]. Thus this mesenchymal cell type served as an excellent positive control for our studies and confirmed, at least in part, previous observations that hPL products can be more effective than FBS at enhancing hBM-MSC osteogenic differentiation [35]. In particular, in the present experimental model, we noticed a variability regarding the mineralization potential compared to FBS, depending on the hPL used (hPL3 and hPL4 *vs* hPL1 and HPL2).

Although reports on the extent of similarity between hMSC and HDF vary [36-38], dermal fibroblasts can be induced to differentiate osteogenically upon transfection of an osteogenic transcription factor [39] or in soft connective tissue diseases with abnormal mineralization [25-40]. We observed that at low CPD neither neonatal nor adult dermal fibroblasts were capable of prompt biomineralization as observed in hBM-MSC, implicating the latter achieve a more genuinely functional specialised extracellular matrix for mineralization [41].

A feature of nHDF was absence of any mineralization over the time course of the experiment when using FBS, confirming previous data [17]. On the contrary, selective mineralization according to hPL source was observed, with uniquely low levels of mineralization with hPL4. This did not simply reflect a functional inadequacy of hPL4, since this source supported high levels of biomineralization in aHDF at high CPD and in hBM-MSC.
In particular, hPL4 consisted of lyophalized platelet lysate from platelet rich plasma combined with human platelet poor plasma (1:1) and this more comprehensive supplement might include inhibitors as well as stimulators of mineralization that combine for more cell type specific biomineralization [42].
The predominant biomineralization phenotype of aHDF was remarkable by being governed less by choice of FBS or hPL supplementation and more by cumulative population doublings. Although exploring molecular mechanisms was beyond the scope of this study, an accelerated and significantly increased von Kossa staining in high CPD versus low CPD aHDF may include changes in expression of enzymes regulating the important inorganic pyrophosphate/phosphate ratio that regulates calcification [17].

In conclusion, we have shown that it is possible to derive unifying *ex vivo* contexts with contrasting hPL-specific biomineralization phenotypes for direct comparison of physiological and pathological biomineralization. Moreover, use of commercial hPL sources can advantageously allow scalable expansion for further studies exploring the relevant underlying molecular mechanisms.

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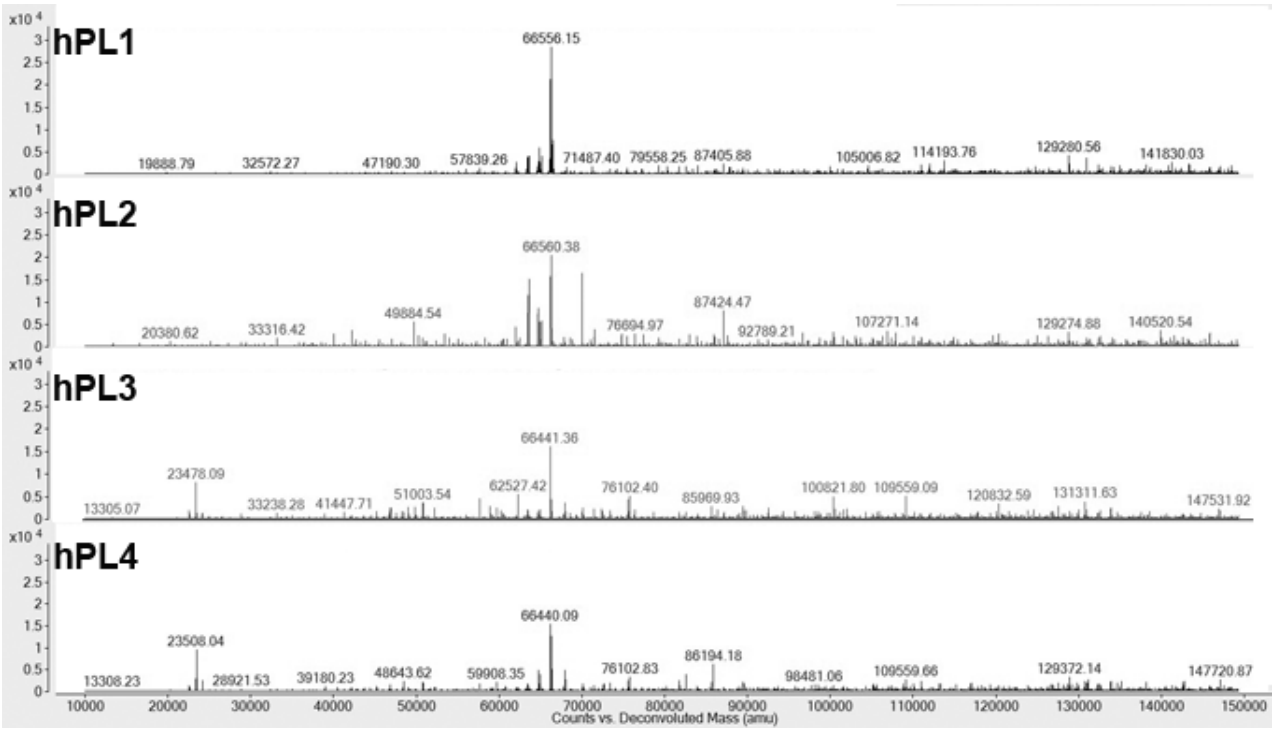

Fig 1. LC-*ESI-Q-TOF profiling*. Representative spectra from each hPL used. Each hPL is characterized by a specific protein profiling indicating a different composition.

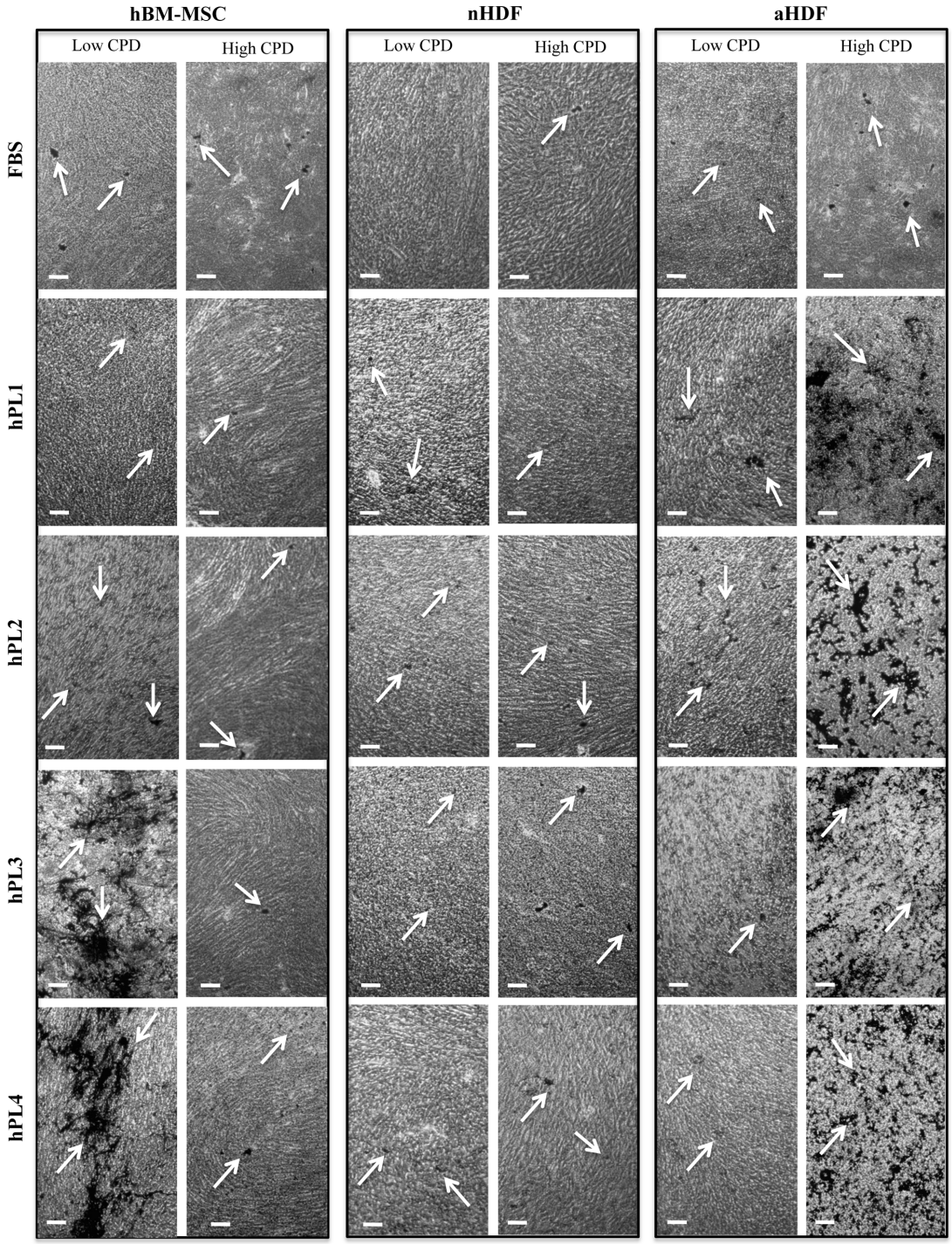

Fig 2. *In vitro* calcification assay. Human bone marrow-derived mesenchymal stromal cells (hBM-MSC), neonatal (nHDF), adult (aHDF) human dermal fibroblasts at low and at high CPD were cultured in calcifying medium for 14 days. Hydroxyapatite deposition (arrows) was assessed by phosphate staining in the extracellular matrix with the von Kossa method. Bar: 120 μm

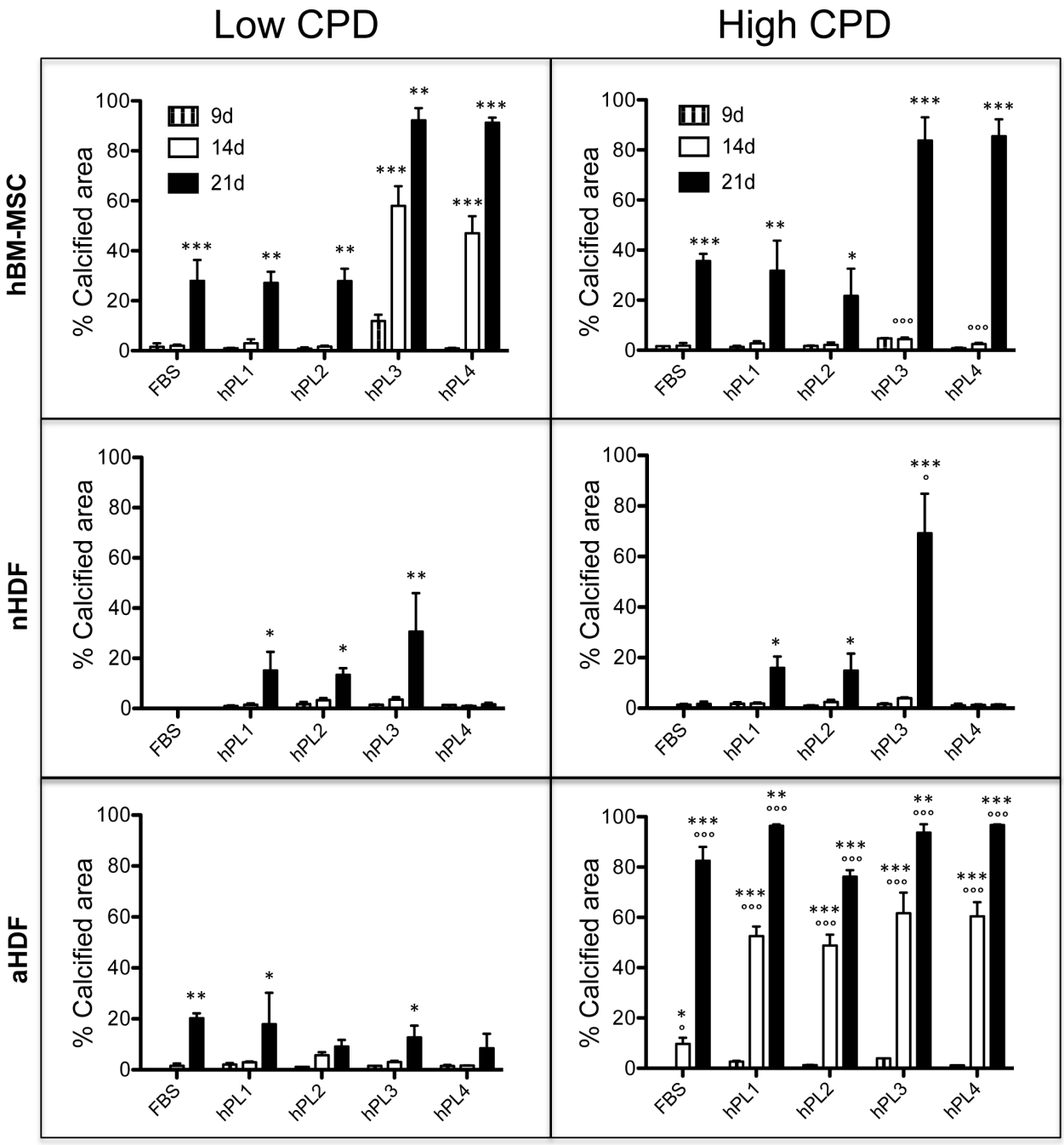

Fig 3. Quantification of mineral deposition. The percentage of calcified areas in human bone marrow-derived mesenchymal stromal cells (hBM-MSC), neonatal (nHDF) and adult (aHDF) human dermal fibroblasts at both low and high CPD, cultured for 9-14-21 days (d) in calcifying media supplemented with FBS or with different hPLs. Data are expressed as mean values ± SEM. *p<0.05;**p< 0.01;***p< 0.001 21d *vs* 14d or 14d *vs* 9d in the same cell line and culture condition °p< 0.05;°°°p< 0.001 high *vs* low CPD in the same cell line and at the same time point and culture condition.

8. Vascular calcification

Vascular calcification is prevalent in ageing and in a number of pathological conditions, and it is now recognized as a strong predictor of cardiovascular events in the general population as well as in diabetic and in end-stage renal disease patients.

Vascular calcification can occur in blood vessels, myocardium and cardiac valves. Calcium phosphate deposits are found in distinct layers of blood vessel.

Vascular calcification has been variously classified according to type and localization. Intimal calcification is seen with advancing age, hypertension, diabetes, dyslipidemia and smoking and takes the form of atherosclerotic vascular disease. It is a patchy and a discontinuous process that involves macrophages and VSMCs in lipid-rich regions (Shrof and Shanahan, 2007).

Medial calcification is common in arteriosclerosis as a complication of aging, diabetes, and end-stage renal disease (Monckeberg JG, 1902; Edmonds ME et al., 1982). Mineral deposits are arranged linearly along resilient strips (Proudfoot and Shanahan, 2001). Medial calcification progresses with age (Elliot and McGrath, 1994) and in severe cases a dense layer of calcium crystals is formed in the center of the tunica media and is delimited, on both sides, by vascular smooth muscle cells and, in some cases, by trabecular bone and osteocytes (Shanahan et al., 1999).

Although a combination of intimal and medial calcification has been observed in patients with chronic kidney desease (CKD), either process may occur independently and, at least in adolescents and young adults with CKD, the involvement is almost exclusively medial (London et al., 2005). Differences between intimal and medial calcification imply different etiologies; however, a common feature of both forms of calcification is the presence of VSMCs (Shanahan et al., 1999; Moe et al., 2002). These cells play a key role in inhibiting calcification in normal vessels, but features associated with vascular pathological environments interfere on their normal function, induce damage, and cause osteogenic phenotypic changes (Tyson et al., 2003; Moe et al., 2002) that favour mineral deposition.

For many years, vascular calcification, was thought to occur predominantly by a passive, unregulated physicochemical mechanism, representing a degenerative, irreversible process associated to aging (Virchow, 1989).

In the last decade, several studies have defined calcification of atherosclerotic lesions as an active process similar to bone formation. Different gene products seem to induce or inhibit the process of ectopic calcification. In particular, matrix Gla-protein, fetuin, osteoprotegerin, and osteopontin may play a very important role on inhibiting mineral deposition in the vasculature. On the other hand alkaline phosphatase, osteocalcin, osteonectin and BMP2 play a very important role on inducing vascular calcification (Cozzolino et al., 2005).

8.1 Mechanisms of vascular calcification

Vascular calcification is currently considered an actively regulated process arising from several different, non- mutually exclusive mechanisms (Speer et al., 2004).

Five different mechanisms for initiating vascular calcification have been proposed. First, human and mouse genetic findings have determined that blood vessels normally express inhibitors of mineralization, such as PPi and matrix Gla-protein, respectively; lack of these molecules ("loss of inhibition") leads to spontaneous vascular calcification and increased mortality (Luo et al., 1997; Rutsch at al., 2003). In the same way, a decrease of fetuin-A, a major inhibitor of apatite found in the circulation, have been recently correlated with elevated mortality in hemodialysis patients (Ketteler et al., 2003). Second, the presence of bone proteins such as osteopontin (Giachelli et al., 1993), osteocalcin (Levy et al., 1983), BMP2 (Bostrom et al., 1993), matrix vesicles (Tanimura et al., 1983), and bone and cartilage formation in calcified vascular lesions (Mohler et al., 2001) suggested that osteogenic mechanisms may also play a role in vascular calcification. Indeed, cells derived from vascular media undergo bone- and cartilage-like phenotypic change and calcify *in vitro* under various conditions (Jono et al., 1998; Tintut et al., 2000). Third, bone turnover leading to release of circulating nucleation complexes has been proposed to explain the link between vascular calcification and osteoporosis in postmenopausal women (Price et al., 2002; Price et al., 2001). Fourth, cell death can provide phospholipid membranous debris and apoptotic bodies that may serve to nucleate apatite, especially in diseases where necrosis and apoptosis are prevalent, such as atherosclerosis (Tanimura et al., 1983; Proudfoot et al., 2000; Schoen et al., 1986). Finally, via thermodynamic mechanisms (sometimes referred as a "passive" mechanisms), elevated Ca and P promote apatite nucleation and crystal growth and would be expected to exacerbate vascular calcification initiated by any of the other mechanisms described above. Furthermore, new evidence suggests that Ca and P may additionally have direct effects on vascular cells thus predisposing to mineralization.

8.2 Progenitors and circulating cells in vascular calcification

The integrity of the endothelium plays a key role in the pathogenesis of vascular diseases, including the development of vascular calcicfication (VC) (Demer et al., 2008; Mashide et al., 2009). To maintain vascular integrity, damaged endothelial cells are replaced by endothelial progenitor cells (EPC), which originate from the bone marrow. In the clinical setting, changes in both the number and functions of EPC have been positively related to an improved capacity for endothelial regeneration, which is inversely associated to cardiovascular risk (Hill et al., 2003; Schmidt-Lucke et al., 2005; Vasa et al., 2007). EPC can be identified not only through their angiogenic capacity but

also by their expression of cell surface markers (Friedrich et al., 2006; Hristov et al., 2004; Medina et al., 2010). It has been recently reported that in patients with coronary atherosclerosis, a high proportion of EPC may exhibit an osteoblast-like phenotype (Eipers et al., 2000; Eghbali-Fatourechi et al., 2007; Long et al., 1995). At present, there is a great interest in evaluating the role of EPC in angiogenesis and in drug therapy monitoring.

On the other hand, a novel way to assess vascular function includes measuring levels of circulating endothelial cells (CEC). Rare in healthy individuals, CEC increase in peripheral blood reflects significant vascular damage and dysfunction. They have been documented in many human diseases, including different types of cancers. The presence of CEC has been recently recognized as a useful marker of vascular damage. Usually absent in the blood of healthy individuals, CEC counts are elevated in diseases hallmarked by the presence of vascular damage, such as sickle cell anemia, acute myocardial infarction, Cytomegalovirus infection, endotoxemia, and neoplastic processes. Current opinion suggests that CEC are cells driven from the intima after vascular insult, and are thus the consequence rather than the initiator of a particular pathology (Goon et al., 2005) (Figure 8). Together with EPC, CEC only represent between 0.01% and 0.0001% of mononuclear cells in normal peripheral blood (Khan et al., 2005), making it very difficult to accurately quantify their numbers.

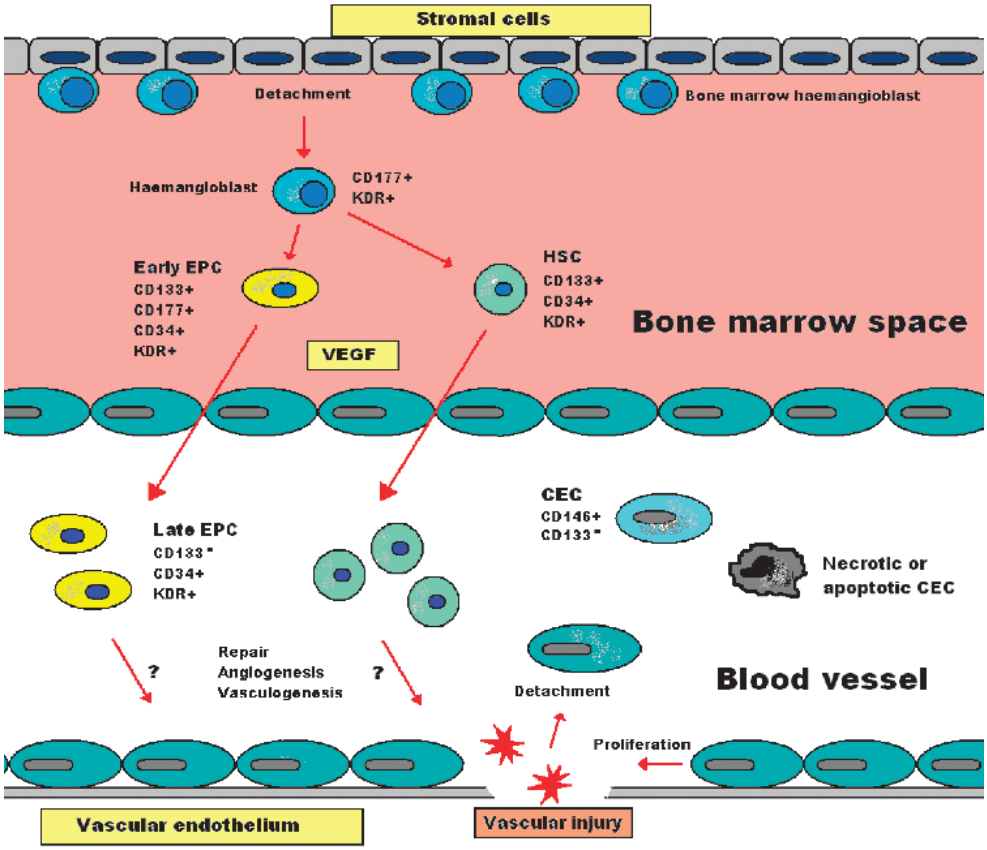

Fig. 8. Schematic representation of CEC, EPC, and hematopoietic stem cells (HSC) in vascular damage, repair and angiogenesis (Goon et al., 2006).

8.2.1 EPC

To detect EPC in peripheral blood, flow cytometry has become the principal method employed.

In the 1999 Asahara and coworkers were the first to isolate EPC in human peripheral blood, using anti- CD34 monoclonal antibodies. An antigen specifically identifying primitive stem cells, CD133, is a novel means to precisely delineate mature (CEC) from immature (EPC) forms (Peichev et al., 2000), although this antigen is only present in human EPC and cannot be applied to mouse EPC (Rafii and Lyden, 2003). Other markers used include vWf, vascular endothelial cadherin (VE-cadherin), vascular endothelial growth factor receptor-2 (VEGFR-KDR) and binding by lectins and acetylated low-density lipoproteins (Peichev et al., 2000; Gehling et al., 2000).

Origin and pathophysiology of EPC

EPC are potentially crucial for neovascularization and may be recruited from bone marrow after tissue ischemia, vascular insult, or tumor growth (Gill et al., 2001; Rafii et al., 2003; Asahara et al., 1999a; Lyden et al., 2001). They possess the ability to migrate, colonize, proliferate, and, ultimately, differentiate into endothelial lineage cells. EPC recruitment and mobilization have been positively correlated with increased levels of angiogenic growth factors such as VEGF (Shintani et

al., 2001). VEGF induces proliferation, differentiation, and chemotaxis of EPC, and is essential for hematopoiesis, angiogenesis, and, ultimately, survival, as evidenced by the nonviability of mouse embryos expressing only a single VEGF allele (Carmeliet et al., 1996). EPC influence cells mainly by interactions with VEGFR-1 and VEGFR-2, both being receptors expressed on hematopoietic stem cells (HSC) and EPC (Rafii et al., 2002).

Other angiogenic growth factors stimulating EPC mobilization include angiopoietin-1, fibroblast growth factor (FGF), stromal-derived factor-1 (SDF-1), placental growth factor (PIGF), and (in mice) macrophage colony-stimulating factor (Asahara et al., 1999b; Takahashi et al., 1999). After mobilization, EPC appear to ‘‘home in’’ and become incorporated into sites of vascular injury and ischemia, with evidence of improvement in the function and viability of tissue (e.g., after acute myocardial infarction) (Kocher et al., 2009). Chemotactic agents responsible for this process include VEGF (Rafii et al., 2002a) and SDF-1 (Yamaguchi et al., 2003), but others may also be involved.

8.2.2 CEC

CEC were first described in the 1970 using methods such as light microscopy, cell morphology, May Grünwald Giemsa staining, and density centrifugation. However none of these methods is capable to identify CEC conclusively due to lack of endothelium specific antibody markers, moreover, the methods were generally too cumbersome. In 1991, monoclonal antibodies to two new cell surface antigens specific to EC (HEC 19 and S-Endo 1) were reported, allowing a more accurate quantification of CEC (George et al., 1992). CEC are generally accepted as cells expressing endothelial markers (e.g., vWf, CD146, and VE-cadherin) in the absence of hematopoietic (CD45 and CD14) and progenitor (CD133) markers. Although CD146 is widely regarded as the principal marker for CEC (mature cell form), it has also been described in trophoblasts, mesenchymal stem cells, periodontal and malignant (prostatic cancer and melanoma) tissues (Goon et al., 2005), and activated lymphocytes (Khan et al., 2005). Consequently, caution in interpreting results with CD146 alone is demanded as cells identified by this marker may indeed be circulating tumor cells or other non-endothelial circulating cell.

Origin and pathophysiology of CEC

The endothelium can be viewed as a membrane-like layer lining the circulatory system, its primary function being the maintenance of vessel wall permeability and integrity. The vascular endothelial cells (EC) layer is relatively quiescent, with an estimated cell turnover period between 47 and 23,000 days, as shown by labeling studies (Hobson and Denekamp, 1984).

Proliferation seems to occur mainly at sites of vasculature branching and turbulent flow. CEC are thought to have ‘‘sloughed off’’ vessel walls, indicating severe endothelial damage (Goon et al., 2005). CEC have been shown to correlate with various endothelial dysfunction and inflammatory markers (Chong et al., 2004; Rajagopalan et al., 2004). Although not fully understood, it would appear that CEC detachment from the endothelium involves multiple factors, such as mechanical injury, alteration of endothelial cellular adhesion molecules (such as integrin aVb3), defective binding to anchoring matrix proteins (such as fibronectin, laminin, or type IV collagen), and cellular apoptosis (Goon et al., 2005; Ruegg et al., 1998). Depending on the disease process, it would seem that the vessel origin of CEC can vary significantly (Solovey et al., 1997; Mancuso et al., 2001; Butthep et al., 2002).

9. VASCULAR CALCIFICATION MODEL

Vascular calcification is characterized by the deposition of calcium-phosphate complexes mostly in the form of hydroxyapatite into the tissue. This condition can be observed during ageing as a complication of age-related diseases (*i.e*. atherosclerosis, diabetes), but also in a number of genetic disorders. Several factors including hypertension, inflammation, oxidized low density lipoproteins, stress, hypercalcemia, hyperphosphatemia and a high calcium-phosphorous ion product can induce and or influence the mineralization. Moreover, it has been demonstrated that vascular smooth muscle cells (VSMC) undergo trans-differentiation into osteoblast-like cells. Nevertheless, there are evidences that osteoblast-like cells are present in peripheral blood, suggesting that also circulating cells may contribute to ectopic calcification. Cells showing chondrocyte/osteoblast-like morphological and biological features might originate from mesenchymal progenitors resident in the vascular wall, from trans-differentiation of mature vascular smooth muscle cells or from circulating cells harbouring a calcifying potential. In particular, many studies focused on the role of circulating endothelial (CEC) and circulating progenitor cell (EPC) in ectopic vascular calcification. To date there are contradictory data on the terminology and the detection methods used for these cells. Therefore, using of new-generation polychromatic flow cytometry we have developed a new protocol to accurately identify these rare circulating cells. Accordingly, we analyzed circulating cells obtained from individuals without any clinical evidence of vascular manifestations and patients affected by peripheral artery complications due to ectopic calcification (***vascular calcification model***) (Federica Boraldi[1], Angelica Bartolomeo[1], Sara De Biasi[2], Stefania Orlando[1], Sonia Costa[1], Andrea Cossarizza[2], Daniela Quaglino[1], J. Translational Medicine *Submitted*).

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High performance flow cytometry analysis of rare circulating cells involved in endothelial dysfunction may disclose future clinical applications.

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Abstract:	Background Circulating endothelial and progenitor cells, although rare in the circulation, are markers of endothelial damage and repair potential, possibly predicting the severity of cardiovascular manifestations. A number of studies highlighted the role of these cells in age-related diseases, including those characterized by ectopic calcification. Nevertheless, their use in clinical practice is still controversial, mainly due to difficulties in finding reproducible and reliable methods for their determination. Methods Circulating CD45-, CD34+, CD133- mature cells (CMC) and CD45dim, CD34bright, CD133+ progenitor cells (CPC) have been therefore investigated by polychromatic high-speed flow cytometry by detecting several antigens (i.e., CD14, CD19, CD45, CD34, CD133) in healthy subjects and in patients with peripheral vascular manifestations associated with ectopic calcification. The expression of markers of endothelial (CD309) or osteogenic differentiation (bone alkaline phosphatase, BAP) has been also evaluated. Results This study shows that: 1) polychromatic flow cytometry represents a fundamental tool to accurately identify rare cells; 2) the balance of CMC/CPC, both positive for CD309, is altered in patients affected by peripheral vascular manifestations, suggesting the occurrence of vascular damage and low repair potential; 3) the increase of circulating cells with a shift towards an osteoblast-like phenotype is observed in the presence of ectopic calcification. Conclusion The differences we have found between healthy subjects and patients suffering of ectopic calcification indicate that this approach may be useful to better evaluate endothelial dysfunction in a clinical context.
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High performance flow cytometry analysis of rare circulating cells involved in endothelial dysfunction may disclose future clinical applications.

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Abstract

Background Circulating endothelial and progenitor cells, although rare in the circulation, are markers of endothelial damage and repair potential, possibly predicting the severity of cardiovascular manifestations. A number of studies highlighted the role of these cells in age-related diseases, including those characterized by ectopic calcification. Nevertheless, their use in clinical practice is still controversial, mainly due to difficulties in finding reproducible and reliable methods for their determination.

Methods Circulating $CD45^-$, $CD34^+$, $CD133^-$ mature cells (CMC) and $CD45^{dim}$, $CD34^{bright}$, $CD133^+$ progenitor cells (CPC) have been therefore investigated by polychromatic high-speed flow cytometry by detecting several antigens (i.e., CD14, CD19, CD45, CD34, CD133) in healthy subjects and in patients with peripheral vascular manifestations associated with ectopic calcification. The expression of markers of endothelial (CD309) or osteogenic differentiation (bone alkaline phosphatase, BAP) has been also evaluated.

Results This study shows that: 1) polychromatic flow cytometry represents a fundamental tool to accurately identify rare cells; 2) the balance of CMC/CPC, both positive for CD309, is altered in patients affected by peripheral vascular manifestations, suggesting the occurrence of vascular damage and low repair potential; 3) the increase of circulating cells with a shift towards an osteoblast-like phenotype is observed in the presence of ectopic calcification.

Conclusion The differences we have found between healthy subjects and patients suffering of ectopic calcification indicate that this approach may be useful to better evaluate endothelial dysfunction in a clinical context.

Key words: Circulating endothelial mature cells, circulating endothelial progenitor cells, flow cytometry, endothelial dysfunction, ectopic calcification

Introduction

Endothelial dysfunction is crucial for the development and progression of many cardiovascular disorders. Therefore, the possibility of using non-invasive techniques to evaluate endothelium damage and repair potential has a relevant clinical value. Circulating endothelial cells consist of mature endothelial cells detaching from the intima monolayer in response to endothelial damages [1]. These cells are detectable in peripheral blood. Indeed, even if are rare in healthy individuals, they can be more abundantly detected in patients with cardiovascular-related complications [2-4], suggesting that they may be taken as indicator of disease severity [5].

When injury or tissue damage occurs, circulating progenitor cells are thought to mobilize from bone marrow into the circulation, homing to sites of tissue repair under the guidance of several signals [6]. To assure an adequate homeostatic tissue control, repair activities should compensate the extent of damage processes, or endothelial dysfunction takes place. Moreover, endothelial and vascular smooth muscle cells can differentiate into osteoblast-type cells [7], thus contributing to ectopic calcification, one of the most frequent complication of the aging vasculature. These cells might originate from resident vascular mesenchymal progenitors, from trans-differentiation of mature vascular smooth muscle cells or from circulating cells with a calcifying potential [8, 9].

Despite the number of studies performed so far, investigating circulating endothelial and progenitor cells is technically challenging and contradictory results are frequently reported, due to discrepancies in terms of terminology and protocols used for the detection of these cells, thus leading to ambiguous conclusions affecting the significance of data in the clinical practice [10].

The present study has been undertaken with the aim to investigate circulating cells in healthy subjects and in patients affected by peripheral vascular manifestation associated to ectopic calcification. Thus, circulating $CD45^{-}$, $CD34^{+}$, $CD133^{-}$ mature cells (CMC) and $CD45^{dim}$, $CD34^{bright}$,$CD133^{+}$

progenitor cells (CPC) were firstly identified by means of a panel of antigens (CD14, CD19, CD45, CD34 and CD133). Then, on these two cell populations, the expression of markers of endothelial (CD309) or osteogenic (bone alkaline phosphatase, BAP) differentiation were searched.

As a model, we have used blood from patients affected by *Pseudoxanthoma elasticum*, a genetic disorder characterized by progressive mineralization of elastic fibres within soft connective tissues, thus causing the premature occurrence of *claudication intermittens* [11, 12]. This approach allows evaluating if the proposed methodology is capable to highlight differences between individuals without any clinical evidence of vascular manifestations and patients affected by peripheral artery complications. Moreover, since these patients are characterized by ectopic calcification, we have also investigated if these circulating cells may exhibit a shift towards an osteoblast-like phenotype.

Methods

Blood sample collection

Up to 33 mL peripheral blood were collected in EDTA-coated tubes from 20 patients affected by *Pseudoxanthoma elasticum* (PXE) (mean age± std: 44±16 yr) and from 22 healthy subjects (47±15 yr). Patients suffered from vascular alterations (*claudication intermittens*, hypertension). Clinical diagnosis of PXE has been molecularly confirmed by demonstrating two causative mutations in the ABCC6 gene. All individuals gave signed informed consent and procedures were performed in accordance with the guidelines of the Helsinki declaration and the Medical Ethical Committee of the University of Modena and Reggio Emilia (#35/15).

Samples were processed immediately after venepuncture. In order to avoid the interference of endothelial cells damaged by needle insertion through the vessel wall, the first 3 mL of blood were discarded[5]. Peripheral blood cells were obtained according to standard protocols. Plasma was centrifuged at 3,500 rpm for 15 min at 4°C and stored immediately at -80°C until analyses.

Flow cytometry analysis

Circulating $CD45^-$, $CD34^+$, $CD133^-$ mature cells (CMC) and $CD45^{dim}$, $CD34^{bright}$,$CD133^+$ progenitor cells (CPC) were evaluated by 4-laser flow cytometry, using a panel of monoclonal antibodies, including those recognizing CD14-APCH7 (Becton Dickinson, Milan, Italy - BD), CD19-APCH7 (BD), Live Dead far red (Life Technologies - Thermo Fisher Scientific), CD45-PE (R&D System, Minneapolis, MN, USA), CD34-PC7 (Beckman Coulter, Milan, Italy), CD133-APC (Miltenyi, Bologna, Italy), CD309-FITC (R&D System) or BAP-FITC (R&D System). Cells were first gated on the basis of forward scatter (FSC) and side scatter (SSC). Doublets were removed by physical parameters. Dead cells, B cells, monocytes and cell debris were removed by the use of electronic gate and the dump channel (containing mAbs against CD19, CD14 and Live Dead). At this point circulating mature and progenitors cells were defined as $CD45^-/CD34^+/CD133^-$ and $CD45^{dim}/CD34^{bright}/CD133^+$, respectively (Fig. 1). These cells were finally analysed for the expression of CD309 (marker of endothelial differentiation) or bone alkaline phosphatase (BAP, osteogenic marker).

Using a novel strategy for the identification of rare events [13], a minimum of 5 million cells per sample was acquired using a 8-parameters Attune Acustic Focusing Flow Cytometer (Applied Biosystems-Thermo Fisher Scientific), equipped with lasers at 488 nm and 634 nm. Data regarding CPC and CMC were then analysed by FlowJo 9.8.3 (Treestar Inc., Ashland, OR) under MacOS 10 [14]. Single staining and Fluorescence Minus One (FMO) controls were performed for all panels to set proper compensation and define positive signals [15].

Quantification of soluble molecules

Plasma levels of vascular endothelial growth factor (VEGF), stromal-derived factor-1alpha (SDF-1α); Interleukin-1β (IL-1β); interleukin-6 (IL-6); tumor necrosis factor (TNF-α) and soluble receptor for advanced glycosylated end products (sRAGE) were determined using commercial ELISA

kit according to manufacturer's instructions (Quantikine - R&D Systems). Measurements were done in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 5.01 for MAC (GraphPad Software, San Diego, CA, USA). Data sets were compared using non-parametric analysis of Mann–Whitney test. P values less than 0.05 were considered statistically significant.

Results

CMC from patients with vascular manifestations show an increased expression of BAP

Circulating progenitor cells ($CD45^{dim}/CD34^{bright}/CD133^{+}$) (CPC) and circulating mature cells ($CD45^{-}/CD34^{+}/CD133^{-}$) (CMC) were identified by a highly sensitive new generation of acoustic flow cytometer capable to analyse up to 35,000 cells per second, and thus $2x10^{7}$ cells in few minutes. These circulating cell populations were similarly represented in patients and in control subjects (data not shown). Therefore, starting from a comparable amount of cells, we have assessed the presence of the endothelial marker of differentiation CD309 and of the osteogenic marker BAP (Fig. 2).

Analysis on CPC showed that, compared to healthy subjects, patients exhibited a similar amount of $CD309^{+}$ cells (Fig. 2a), along with a small but not significant increase of BAP^{+} cells (Fig. 2b). However, the ratio of $BAP^{+}/CD309^{+}$, taken as an indicator of the phenotypic shift of CPC towards an osteoblast-like phenotype, highlighted a two-fold increase in patients versus healthy subjects (Fig. 2c).

We also investigated CMC positive for CD309 or for BAP. Patients showed an increase of the percentage of $CD309^{+}$ cells, when compared to healthy subjects (Fig. 2d). Moreover, the increased percentage of BAP^{+} cells detected in patients demonstrates an osteoblast-like phenotypic shift (Fig. 2e). Finally, the ratio between $CD309^{+}$ on CPC and $CD309^{+}$ on CMC was significantly lower in patients than in healthy subjects (Fig. 2f).

Patients with vascular manifestations showed increased plasma levels of VEGF

In order to correlate the occurrence of vascular damages to typical inflammatory markers, cytokines as such IL-1β, IL-6 and TNF-α, growth factors as SDF-1α and VEGF as well as the soluble factor sRAGE were quantified in plasma from controls and in patients. No significant differences were observed as far as SDF-1α, IL-1β, IL-6, TNF-α and sRAGE expression were concerned (Fig. 3a-e). In contrast, VEGF levels were significantly higher in patients than in healthy subjects (Fig. 3f).

Discussion

Several approaches have been used to identify circulating rare cells and, even though flow cytometry appears as the most promising and rapid technique, no consistent and conclusive data are reported so far, mainly because of the limited number of antigens detected as well as their expression on overlapping phenotypes [16]. It has to be underlined, indeed, that the identification of circulating rare cells (i.e., those representing 0.0001 to 0.01% of peripheral blood cells), requires the acquisition of a large number of events, typically of the order of several millions, and that the amount of some antigens on the cell surface can be so low that conventional flow cytometers are not able to identify "dim" and "bright" populations according to the intensity of fluorescence of a given antigen-bound antibody.

To cope with the aforementioned problems, the cytometer used in the present study is the first one that applies ultrasonic waves (over 2 MHz, similar to those used in medical imaging) rather than hydrodynamic forces to position cells into a single focused line along the central axis of a capillary. Keeping cells within a confined focal point is a crucial requirement for the consistent excitation of conjugated fluorochromes, for maintaining the same sample speed at all flow rates, and especially for reaching an incredible speed of acquisition. This approach allows a relatively easy

analysis of rare events, with a speed that is about 50-100 times higher than that typically used in this field. Finally, it has to be underlined that, from a statistical point of view, the analysis of a dramatically high number of events is crucial to obtain the accuracy that is required for the identification and quantification of rare cells. Based on this new technology and on the knowledge on specific marker studies [17], we were able to accurately identify cells discriminating between circulating progenitor cells ($CD45^{dim}/CD34^{bright}/CD133^{+}$) and circulating mature cells ($CD45^{-}/CD34^{+}/CD133^{-}$).

No changes have been observed in the number of CPC in controls and in patients. These data are in agreement with the similar amount of plasma SDF-1α, a growth factor responsible for the mobilization of stem and progenitors cells from bone marrow environment to the blood [18].

The increased number of circulating mature endothelial cells detached from the intima of the vessel wall ($CD309^{+}$ on CMC) in patients with vascular clinical manifestations, compared to healthy subjects, confirms the role of these cells as markers of endothelial damage [2, 4]. Moreover, the different ratio of CPC and CMC, both positive for CD309, may indicate that in patients there is a reduced vascular repair potential indicative of endothelial dysfunction.

The ratio of bone (BAP) versus endothelial (CD309) marker expression highlights that, in patients with ectopic calcification, CPC undergo a shift towards an osteogenic phenotype. It could be suggested that, when progenitor cells are recruited to sites of vascular damage, they could promote vascular calcification. Moreover, in patients' peripheral blood, an increase of BAP^{+} on CMC was also found. Ectopic calcification is a frequent complication of aging vessels, significantly contributing to cardiovascular manifestations [19]. Therefore, the possibility to evaluate the osteogenic potential of circulating cells, in addition to evidence of endothelial dysfunction, represents a valuable non-invasive tool for a better management of the aging population at increased risk of cardiovascular events.

It is also known that angiogenesis contributes to aberrant mineralization since new vessels can act as a conduit for osteo-progenitor cells including both circulating progenitor cells and pericytes present within vessels [7]. The increased amount of plasma VEGF measured in patients characterized by ectopic calcification suggests that VEGF, favoring the angiogenic process, may contribute to ectopic calcification by recruiting osteoblast-like cells at specific sites.

In order to exclude that calcification in these patients was the consequence of a generalized inflammatory process, we measured the amount of inflammatory cytokines as IL-1β, IL-6, TGF-β, TNF-α and s-RAGE [20-22]. No changes were detected for these soluble factors, further demonstrating that ectopic calcification may take place also in the absence of an inflammatory condition.

Conclusions

Although limited by the relatively small number of subjects, this study represents a proof of concept that: 1) the use of new-generation polychromatic flow cytometry is crucial to accurately identify rare cells; 2) altered $CD309^+$ on CMC/$CD309^+$ on CPC balance, suggestive of vascular damage and low repair potential, can be revealed in patients with disease-affected peripheral vessels also in the absence of a clinically relevant inflammatory condition; 3) an increase of circulating cells with a shift towards an osteoblast-like phenotype might be related to the presence of ectopic calcification. Thus, our results may pave the way to future studies on a larger cohort of individuals for the potential use of this approach to better evaluate endothelial dysfunction in a clinical context.

List of abbreviations

CMC: circulating $CD45^-$, $CD34^+$, $CD133^-$ mature cells **CPC**: circulating $CD45^{dim}$, $CD34^{bright}$, $CD133^+$ progenitor cells **BAP**: bone alkaline phosphatase **CD309**: vascular endothelial growth factor receptor-2 **PXE**: Pseudoxanthoma elasticum **VEGF**: vascular endothelial growth factor **SDF-1α:**stromal-derived

factor-1 alpha **IL-1:** Interleukin-1 **IL-6**: interleukin-6 **TNF-** α: tumor necrosis factor **sRAGE**: soluble receptor for advanced glycosylated end products.

Declarations

Competing interests

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Author's contributions

FB and DQ conceived the study, AB, SDB and SO carried out the experiments, SC performed the molecular characterization of patients; FB and AC were involved in data interpretation, FB and DQ wrote and revised the manuscript.

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Figure Legends

Fig. 1 Gating strategy for the identification of circulating ($CD45^{dim}$, $CD34^{bright}$, $CD133^{+}$) progenitor cells (CPC) and circulating ($CD45^{-}$, $CD34^{+}$, $CD133^{-}$) mature cells (CMC). (**a-b)** Peripheral blood mononuclear cells (PBMCs) were gated according to physical parameters. **(c)** Debris, B cells, monocytes and dead cells were removed by the use of electronic gate and the dump channel (containing mAbs against CD19, CD14 and a viability marker, i.e. Live Dead). CMC and CPC were identified on the basis of the expression of CD34, CD45 and CD133. **(d-e)** CMC were defined as $CD45^{-}$, $CD34^{+}$, $CD133^{-}$. **(d-f)** CPC were defined as $CD45^{dim}$, $CD34^{bright}$, $CD133^{+}$.

Fig. 2 Phenotypic characterization of $CD45^{-}$, $CD34^{+}$, $CD133^{-}$ mature cells (CMC) and $CD45^{dim}$, $CD34^{bright}$, $CD133^{+}$ progenitor cells (CPC) in healthy subjects (CTR) and in patients (PT). Histograms show the percentage of $CD309^{+}$ (marker of endothelial differentiation) or BAP^{+} (osteogenic marker) on CPC in CTR and in PT (**a, b**). The different $BAP^{+}/CD309^{+}$ ratio on CPC suggests that circulating progenitor cells in patients undergo a shift towards an osteogenic phenotype (**c**). Histograms show the percentage of $CD309^{+}$ or BAP^{+} on CMC in CTR and in PT (**d, e**). The different $CD309^{+}$ on CPC/$CD309^{+}$ on CMC ratio suggests that patients have a lower vascular repair potential (**f**). Values are shown as mean ± SD. *p<0.05

Fig. 3 ELISA test. Amount of stromal-derived factor-1alpha (SDF-1α) (**a**); interleukin-1β (IL-1β) (**b**); interleukin-6 (IL-6) (**c**); tumour necrosis factor-α (TNF-α) (**d**); soluble receptor for advanced glycosylated end products (sRAGE) (**e**) and vascular endothelial growth factor (VEGF) (**f**) were determined in plasma of healthy subjects (CTR) and of patients (PT). Values are shown as mean ± SD. *p<0.05

Figure 1

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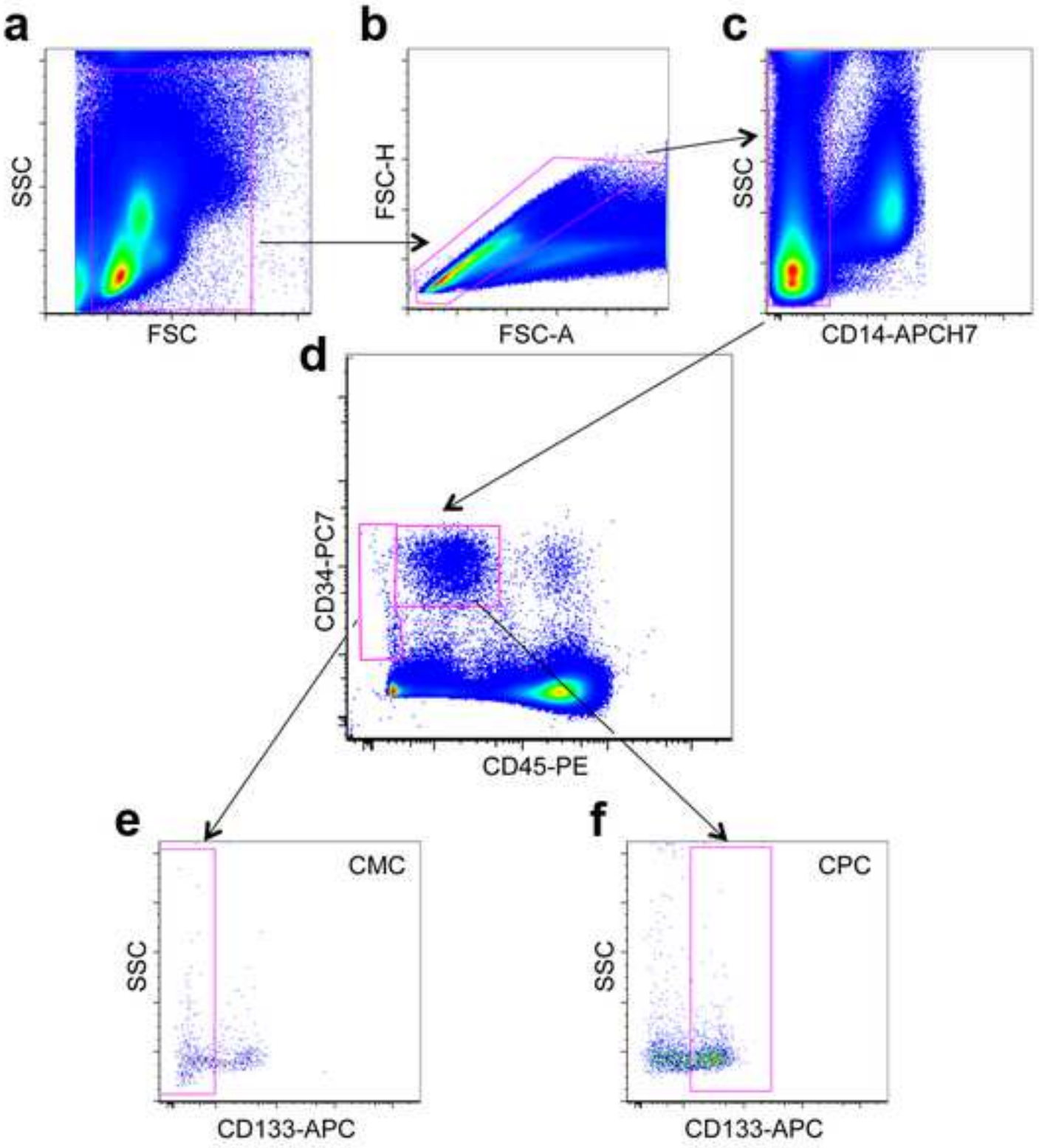

Figure 2

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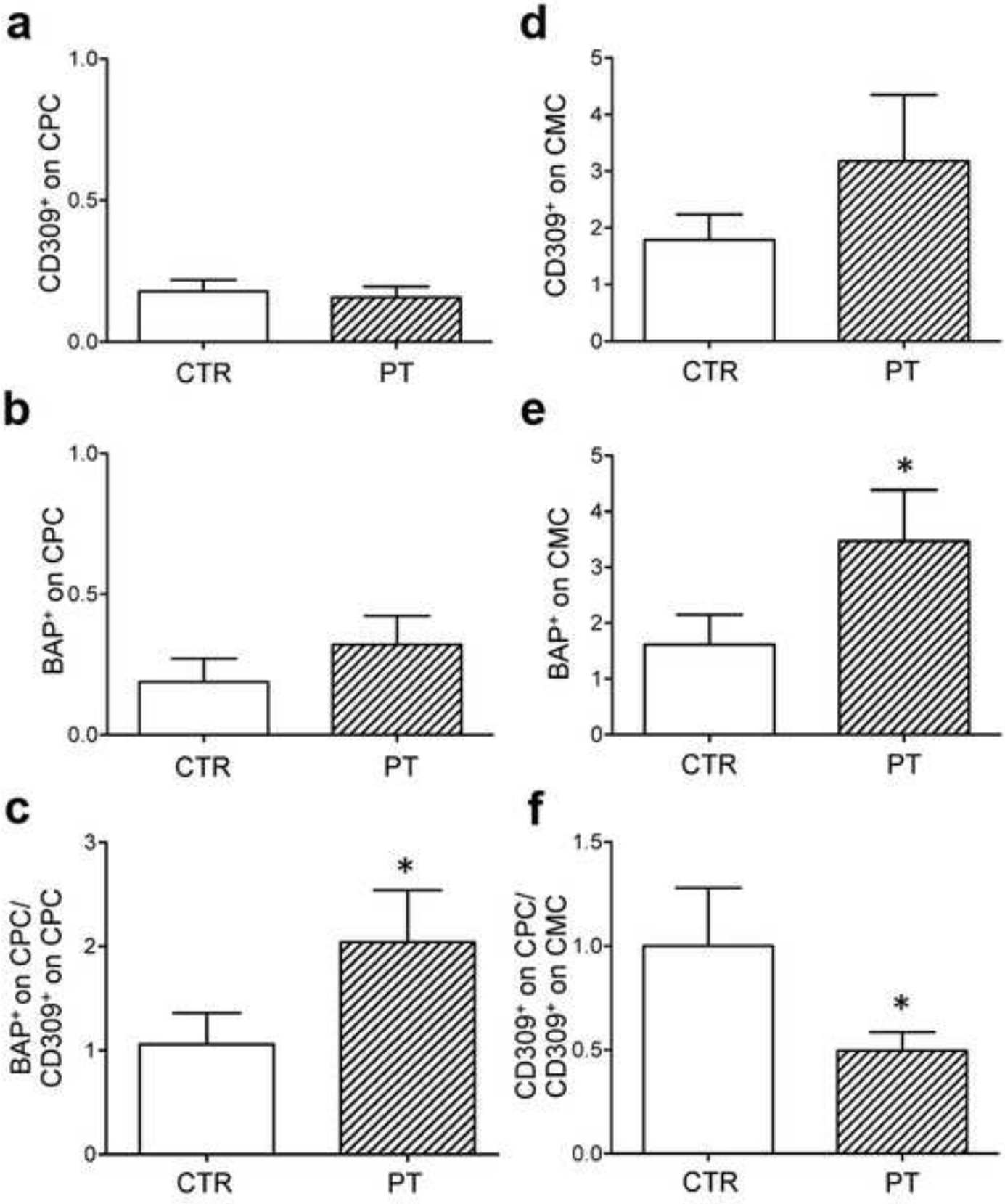

Figure 3

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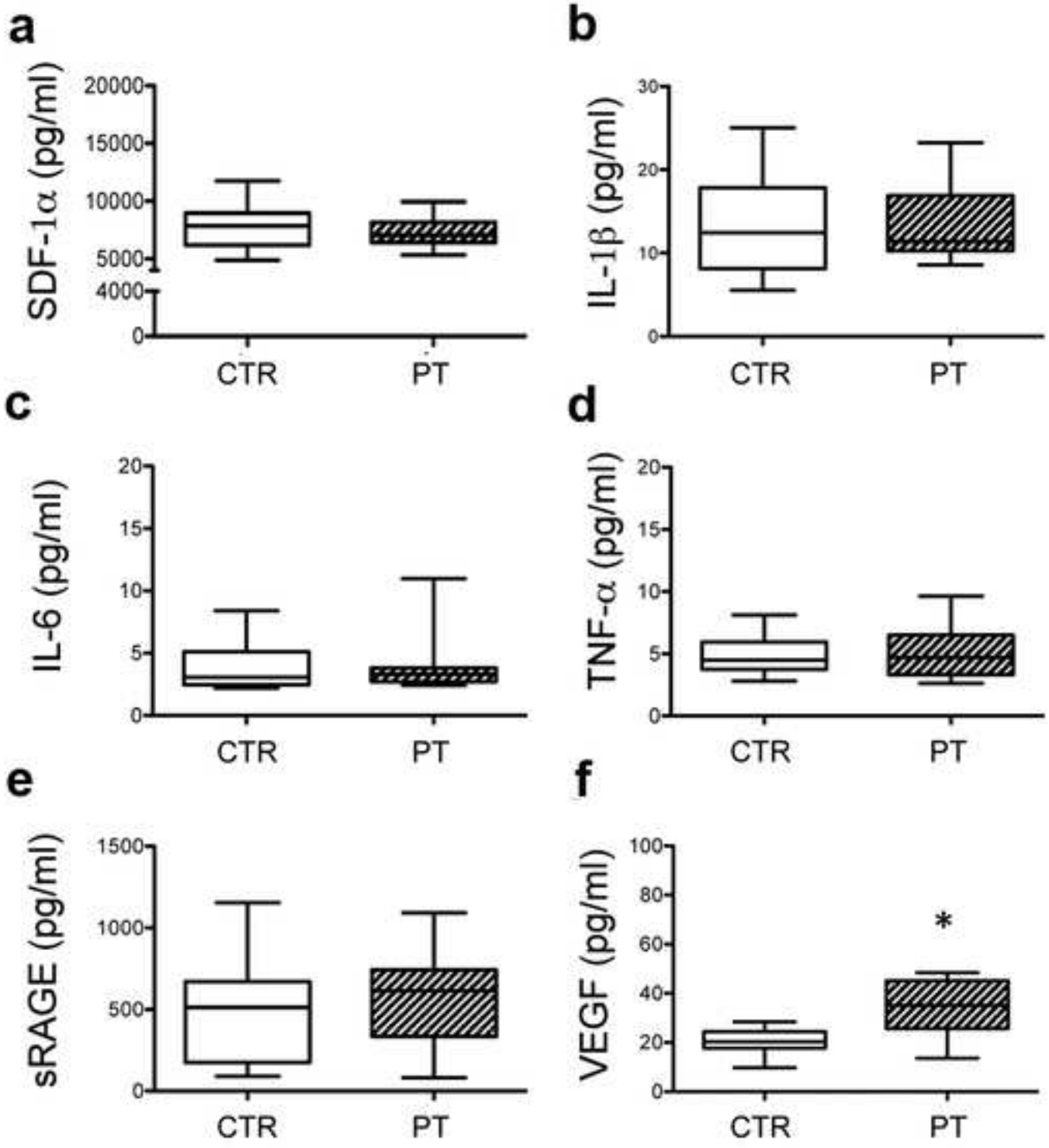

10. Three-dimensional scaffold

Tissue-engineering scaffolds provide the structural support for cell invasion and growth and eventually direct and maintain cell differentiation *in vivo* (Shin et al., 2003). It is becoming increasingly crucial in biomaterial-based approaches for regenerative medicine and in cell biology to study cell-matrix interactions in a three-dimensional (3D) environment.

Tissue engineering aims to develop biological substitutes that replace or restore the function of damaged tissues (Langer and Vacanti, 1993). Very important for cell behaviour is the scaffold morphological architecture. In general, scaffold porosity dictates the surface area for initial cell seeding, while pore size and its interconnectivity affect cell infiltration and migration, as well as cell morphology and differentiation. Closed pores can reduce the path length for the diffusion of gases to the core of the scaffold, whereas open pores are the key to ensuring cell proliferation and nutrient diffusion. It is also believed that the degree of interconnectivity rather than pore size has a greater influence on osteoconduction (Gorgieva et al., 2014).

Compared to synthetic polymers, protein- and polysaccharide-based polymers are more favourable platforms for cell colonization and growth; reducing post-degradation tissue toxicity or inflammatory host responses (Hubbell, 1995). These can be extracted from tissue extracellular matrix (*e.g*. collagen, glycosaminoglycan, hyaluronic acid, elastin) or derived from non-mammalian biological sources (*e.g*. silk fibroin, chitosan, plant cellulose). This large variety of biofunctional molecules can be processed in different forms (*e.g*. hydrogels, freeze-dried sponges, electrospun mats), in order to support cell adhesion and proliferation and to be subsequently metabolized by endogenous enzymes without significant adverse effects on host tissues (Lee et al., 2001; Li, 2006). Collagen type I-based hydrogels have been extensively used as 3D culture environments, upon fibrillization of purified collagen solutions (Bell et al., 1979). Reconstituted collagen gels allow the 3D homogeneous distribution of cells by direct injection and provide a distinctive cell growth environment (Stella et al., 2010). The complex interactions of cells with their microenvironment affect cell decisions *in vivo* as well as *in vitro*. Various approaches have been investigated to tailor the structural, functional and mechanical properties of collagen matrices, including reinforcement with synthetic polymers for the reconstruction of a number of tissues, such as skin (Chen et al., 2005), cartilage (Kawazoe et al., 2010), ligament (Chen et al., 2004) and bladder (Nakanishi et al., 2003). Furthermore, other naturally derived polymers (e.g. glycosaminoglycan, chitosan and elastin) have been used in association with collagen for skin (Ma et al., 2003), cartilage (Farrell et al., 2006), bone (Wang and Stegemann, 2010) and vascular (Simionescu et al., 2006) constructs. Alternatively, plastic compression (PC) has been proposed as a method to produce tissue equivalent dense collagen (DC) gels by the rapid expulsion of most of the water content (Brown et al., 2005),

resulting in a nanofibrillar architecture with controlled collagen density and enhanced strength and modulus values (Abou Neel et al., 2006).

In the last 40 years since Elsdale and Bard's analysis (1972) of fibroblast culture in collagen gels, we have moved far beyond the concept that such 3D fibril network systems are better models than monolayer cultures. New models are developed to understand cell-mechanical function in connective tissues, and in particular collagen material has become progressively more important, being engineered to mimic more complex aspects of native extracellular matrix structure. These have included collagen fibril density, alignment and hierarchical structure, controlling material stiffness and anisotropy. Fibril-forming collagens, *i.e.*, types I, III, V, XI, XXIV and XXVII, are interesting candidates for the reconstitution of a bio-analogue 3D ECM *in vitro* (Franke et al., 2014) and for the investigation of normal and pathological fibroblast mechano-functions (Brown et al., 2013).

10.1 Three-dimensional calcifield models

In the last years many studies are performed to understand the process of physiological and pathological biomineralization, using *in vivo* and *in vitro* 2D or 3D models.

Collagen mineralization *in vitro* is of great interest for the understanding of the mechanisms underlying the mineralization *in vivo* as well as for the synthesis of improved bone grafts. It is known that mineralization *in vivo* is controlled by some proteins which are found in bone, the so-called noncollagenous proteins (Boskey, 1989). Gerstenfeld *et al* (1989) have showed through *in vitro* studies of tissue formation with chicken osteoblast cultures that bone formation can be considered as a multistage process. First a collagen template is grown, and then noncollagenous proteins are formed, which finally control calcium phosphate crystallization. Though the biological formation of calcium phosphate in a collagen matrix has not been completely understood, biomineralization should take place with a highly regulated cellular control to result in a well-defined structure and composition. Alkaline phosphatase, for istance, acts most likely on organic phosphate to release phosphate ions, which are subsequently captured by Ca^{2+} ions at nucleation sites of the protein. In the last years a number of studies on the biomineralization process have been performed *in vitro*. Yamauchi *et al* (2004) proposed a collagen/calcium phosphate multilayer sheet (2-10 layers), which consisted of alternately cumulated collagen and calcium phosphate layers, with the thickness of 6-8 μm in each layer. In this case the inorganic layer was mineralized by means of alkaline phosphatase. Bradt and coworkers in 1999, to find new ways for the synthesis of improved bone implant materials, produced a biomimetic mineralization of collagen by combining fibril assembly and calcium phosphate formation with addition of polyaspartate to the reaction mixture.

Uchihasci *et al* (2013) produced a system to study osteoblasts migration and differentation in which cells were seeded on a layer of solidified type I collagen gel and covered with a osteogenic medium (αMEM containing β-glycerophosphate and ascorbic acid). Ghezzi *et al* (2015) stabilized their efforts to obtain a multilayered dense collagen-silk fibroin hybrid, a platform for mesenchymal stem cell differentiation towards chondrogenic and osteogenic lineages.

Though various methods have been reported for the preparation of inorganic/organic composite materials such as the mineralizations using alkaline phosphates (Banks et al., 1997; Doi et al., 1996), self-assembled surfactant- and polymer-matrices (Breulmann et al., 2000; Heywood et al., 1994), soaking polymer materials (sheet, sponge and gel) in a simulated body liquid (Sato et al., 2000; Murphy et al., 2000) and alternate soaking in aqueous solutions of Na^2HPO^4 and $CaCl^2$ (Chen et al., 2001; Taguchi et al., 2001), there isn't, to date, a method able to develop a homogeneously mineralized collagen gel, in wich crystal deposits are randomly distributed into and in all part of the organic matrix. Only in this way will be possible to mimic as close as the physiological and physicochemical conditions of mineralization process and to study the behaviour of mesenchymal cells involved in biomineralization.

11. 3D CALCIFIED MODEL

Nowadays it is known that biomineralization should take place under the control of highly regulated cellular mechanisms resulting in a well-defined structure and composition of calcium-phosphate crystals. A number of studies have been reported using *in vitro* tree-dimensional (3D) models to investigate the effect of a calcified environment on mesenchymal cells. In particular there are many studies that have developed an *in vitro* 3D calcified gel, but each of these models has some puculiar features and possibly some limitation. In some cases, calcification is not homogemeous or it is the consequence of a passive deposition of precipitates, or cells are spread only on the surface of the gel and not inside a 3D structure. Accordingly, to better mimic the *in vivo* calcified environment allowing to better investigate cellular behavior, we have developed an *in vitro* 3D system where it is possible to induce, in a short time, an enzymatically-induced deposition of mineral precipitates. Inside the mineralized collagen matrix it is possible to culture mesenchymal cells and to study their behaviour (***3D calcified model***) (*Manuscript in preparation*).

Matrix characteristics modify the behaviour of fibroblasts embedded within a 3D enzymatically mineralized collagen gel.

Introduction

Formation of hard connective tissue, such as dentin, bone and cementum, involves calcium phosphate deposition within a collagen matrix. Apparently, similar mechanisms may take place also in the aberrant calcification of soft connective tissues, nevertheless regulatory mechanisms, especially in pathologic conditions, are still elusive.

Therefore, exploiting strategies capable to regulate mineral deposition demands a better understanding of cell behaviour in a calcified context and requires the development of simple and reproducible models. Due to the complexity of *in vivo* biomineralization, different *in vitro* models are frequently applied, most of them providing pro-mineralizing factors' supplementation into the culture medium.

Despite the use of a variety of natural macromolecules and/or synthetic polymers as organic matrices for hydroxyapatite mineralization, collagen type I is the most recurrent embedding substrate for cell encapsulation due to its biocompatibility and similarity to native extracellular matrix [1,2]. Moreover, mineralized collagen gels, mimicking bone composition, have been extensively studied for bone tissue engineering applications [3,4]. It is well known that collagen *per se* does not induce mineral formation, requiring the presence of ions/salts [5], of non collagenous proteins [6) and/or of amorphous calcium phosphate [7]. Nevertheless, an appropriate collagen structure is necessary to guide crystal growth and organization [5] allowing proper collagen-mineral interactions, infiltration of calcium and phosphate within collagen fibrils, hydroxyapatite nucleation [8-10]. Collagen gel mineralization has been either obtained by incorporating hydroxyapatite [11,12], by seeding cells cultured for several days in the presence of pro-osteogenic supplements as β-glycerophosphate and ascorbic acid [13-15], or by preparing collagen/calcium phosphate multilayers where mineralization is the result of enzymatic reactions [16] and cells are spread on the matrix surface. Interestingly, stem cells (i.e. dental pulp stem cells, bone marrow mesenchymal stem cells or adipose stem cells) as well as osteoblasts or smooth muscle cells have been widely investigated, whereas other cell types (i.e. fibroblasts) have been captured little attention, even though they regulate connective tissue biosynthesis and organization and have been associated to a number of pathologic conditions due to ectopic calcification [17].

The aim of this study was to produce enzymatically mineralized collagen gels in which cells (human dermal fibroblasts-HDF) were embedded within the 3D structure.

To achieve this goal, calcium salts ($CaCl_2$) and β-glycerophosphate (β-GP), as a source of organic phosphate, were mixed to the collagen solution in the presence of alkaline phosphatase (ALP) [18,19], the enzyme that promotes phosphate (P) cleavage from organic phosphate-containing substrates, thus releasing P capable to react with calcium ions to form mineral deposits (Fig. 1). Human dermal fibroblasts were added and mixed with the collagen solution before gelification. Since collagen polymerization is sensitive to a number of variables as collagen concentration, pH, temperature or presence of other matrix components (i.e. glycosaminoglycans) [20-23], all experimental conditions were accurately controlled. Since magnesium has been suggested to interfere with mineral deposition possibly preventing ectopic calcification [24], but it has been also suggested to be a modifier of collagen assembly [25], $MgCl_2$ was added to the collagen solution in a set of experiments (Fig. 1).

We determined by complementary morphological approaches: i) presence and distribution of mineral deposits within the collagen gel as a result of ALP activity, 2) role of magnesium supplementation on collagen gel assembly and calcification, 3) cell viability and behaviour of human dermal fibroblasts embedded within the 3D mineralized collagen gel.

Results

Light and environmental scanning electron microscopy reveal a different structure of collagen gels

Sections of paraffin embedded collagen gels were stained with Anilin blue and observed by light microscopy (Fig. 2). In particular, in condition #A and #B, collagen fibrils were very similar in size and in organization forming a loose 3D structure. In condition #C, fibrils appeared organized in a more compact and intricate network, whereas a completely different 3D organization was noted in condition #D, where $MgCl_2$ was added. Surprisingly, fibrils were larger and less dispersed providing a porous structure with larger empty areas between fibrils.

To better visualize the characteristics of collagen gels, samples in the four different experimental conditions were also observed by ESEM (Fig. 3). Moreover, since collagen gels represent suitable 3D substrates to be populated by cells, thus requiring supplementation of serum factors, morphological observations were performed after placing the gels in the absence/presence of FBS, (Fig. 3 left and right column, respectively).

As expected, the fibrillar structural organization of collagen gels in condition #A and #B was very similar. For condition #C, the fibrils are substantially similar in diameter, but their organization was less defined compared to the other experimental conditions. Variation in the mean size of collagen fibrils was not significant. By contrast, in condition #D, there were striking differences in the

morphology of the fibrils whose diameter was significantly larger compared to previous samples. Magnesium chloride was added at concentrations below 100 mM, because higher concentrations enhance collagen solubility and produce flat collagen [25]. Moreover, in a set of preliminary experiments we have tested $MgCl_2$ supplementation ranging from 0.6 mM up to 62 mM, and changes in the organization and structure of collagen fibrils were dose-dependent (data not shown).
By this technique, magnification does not allow to asses if fibrils are laterally fused into larger structures or individual fibrils are simply adjacent one to the other. Observation by scanning transmission electron microscopy seems to confirm the latter hypothesis (suppl. Fig. 1). Interestingly, the presence of FBS, added to the medium after gel polymerization, causes an apparently more dense matrix, possibly due to serum proteins interacting with collagen fibrils and remaining embedded within the 3D structure. To be noted that in condition #C, the presence of FBS rendered gels as murky networks preventing to discriminate single collagen fibrils.

Mineral deposition in collagen gels requires ALP activity

Mineral deposition was evaluated on collagen gel sections stained with the von Kossa method and observed by light microscopy (Fig. 4).
In condition #A and #B, sections were devoid of the typical brown staining associated to phosphate deposits, whereas collagen gels containing ALP (condition #C and #D) were both positive, confirming that P is actually cleaved from substrates and is available to form complexes with calcium. As an additional positive control we have prepared a collagen gel in which mineralization was obtained by salt precipitation after addition of phosphate (3mM) at the same concentrations used in *in vitro* smooth muscle cell models. Heavy mineralization is rapidly obtained, however fibroblast viability appeared significantly reduced and gel retraction stopped at 24 h (suppl. Fig. 2).
To confirm the presence of mineral deposits into collagen gels, we have analysed all samples by ESEM associated with a detector for microanalysis. In particular, Fig. 4 shows the distribution of mineral deposits and the corresponding EDS spectra. Gels in condition #A and #B were devoid of mineral deposits and, consistently, the P peak was absent, whereas in condition #C and #D globular deposits were present within the gel, their distribution being rather homogeneous. EDS analysis on these areas showed Ca and P peaks, the latter becoming available due to ALP activity. To be noted that in condition #D the occurrence of a peak corresponding to Mg can be also detected. Although this finding confirms the addition of Mg to the collagen mix, it cannot demonstrate if magnesium is present or not in combination with phosphate.
Finally, in all samples the peak corresponding to Si was present, since glass slides were used to immobilize collagen gel during analyses.

Homogeneous distribution but different morphology of HDF within collagen gels

The morphology and the distribution of HDF within collagen gels were assessed by light microscopy. In all conditions cells appeared homogeneously distributed within the 3D gel structure, however, in condition #A, #B and #C, HDF exhibited an elongated shape with extended cytoplasmic protrusions, whereas in condition #D the cellular body was more globular with thin branches of various length (Fig. 5).

Enzymatically mineralized collagen gel is cytocompatible

Live/dead fluorescence microscopy of HDF embedded within collagen gels is shown in Fig. 6. Viable cells, stained green, are visible in all conditions after 48h in culture. As a general observation, there is not great difference between gels with or without ALP. Few dead cells can be seen in all samples (Fig. 6).

Because collagen gels are characterized by a different structure and by the absence/presence of mineral deposits, thus possibly affecting cell behaviour, the ability of HDF to retract collagen gels has been evaluated.

As expected, in the absence of serum factors, HDF were not able to contract the gel in any condition (data not shown), confirming that serum components are necessary for retraction. In the presence of serum, after 3h and 6h in culture, collagen gel diameters did not change significantly compared to the initial phase of the experiments. At 24h and 48 h in culture, reduction of gel diameters varied depending on the experimental condition.

In particular at 48h, collagen gels without mineral deposits (#A; #B) were retracted up to 25% of their original diameter. A reduction of 6% to 35% of the original gel size was observed for condition #C and #D, respectively.

Discussion

To better understand the mechanisms regulating the calcification process, either in physiological and/or in pathological conditions, a number of *in vitro* culture systems have been already described, including a few in which mineralization is obtained by addition of alkaline phosphatase during gel preparation [18,19]. The novelty of the present study is that cells are not prepared separately from the mineralized collagen gel, but are added prior to polymerization resulting embedded within the 3D structure of the gel. Moreover, the most frequently used cell lines are represented by osteoblast-like cells [26] and dental pulp stem cells [27], i.e. cells that are predisposed to produce and to be in contact with a calcified extracellular matrix, or by vascular smooth muscle cells that are known to

shift towards an osteoblast-like phenotype [28] Although dermal fibroblasts play a key role in soft connective tissue homeostasis, can be involved in ectopic calcification and, if cultured in 2D system for at least three weeks in the presence of pro-mineralizing factors, can produce a calcified matrix [29,30], to our knowledge no data are available on cell viability and behaviour when these cells are embedded within a calcified matrix.

In the present study calcification was enzymatically induced by the presence of ALP as demonstrated morphologically and by microanalysis. Results demonstrate that fibroblasts, although in a non-physiologic context, can adapt themselves to this mineralized environment.

Moreover, collagen gel structure reflects the experimental condition and in particular the presence of magnesium. Beside variation in collagen concentration and in the number of loaded cells that can modify the structure/porosity of collagen gels [31,32], salts (namely divalent anions and cations) may also influence macromolecular aggregates [33]. Proteins fold into specific and functional three-dimensional structures as the result of specific interactions between amino acid chemical functional groups and the surrounding solvent.

We have investigated the effect of magnesium since there is an increasing interest in this ion as a component of biomaterials used for bone replacement [34] or as a possible therapeutic agent capable to counteract ectopic calcification [24]. Interestingly, in the presence of magnesium, we have demonstrated positivity to von Kossa staining. ALP is not directly affected by magnesium, since different concentration of magnesium do not modify *in vitro* the enzymatic activity of ALP (personal observations). Moreover, recent data have excluded a physicochemical role of Mg^{2+} in inhibiting crystal growth or in altering calcium phosphate crystal composition or structure in an *in vitro* model of vascular smooth muscle cells [35]. By contrast, it has been reported that Mg^{2+} salts can modify collagen assemblies [25]. Consistently, we have noticed a striking effect of $MgCl_2$ on size and organization of collagen fibrils. Formation of a different porous structure could impact on fibroblast's behaviour and cell morphology [36]. Gel retraction, for instance, is dependent on gel structure, being lower in the presence of a dense matrix, as in condition #C, and higher in the presence of large pores, as in condition #D, as already observed by other authors [37]. Moreover, in condition #D cells were less elongated compared to other experimental condition, but collagen gel was more retracted. Previous studies suggested that in fibroblasts a round shape may result from the inability of the collagen matrix to resist the force of cell contraction [38;39]. Additionally, Tamariz *et al.* [40] suggest that adhesive interactions of dendritic extensions was an indication of local remodelling of the collagen matrix, while the stellate/bipolar morphology was a consequence of reorganization and simplification of cell extensions during global remodelling whole gel.

In conclusion, this study demonstrates that in a short time an enzymatically mineralized collagen

gel can be prepared in which mineral deposits and viable cells are homogeneously distributed. The effect of $MgCl_2$ on collagen fibril assembly and organization highlights the importance of ion concentration on matrix structural characteristics and consequently on cell behaviour. The development of structurally different 3D mineralized collagen matrices together with the use of fibroblasts, as a prototype of soft connective tissue mesenchymal cell, may pave new ways for the study of ectopic calcification.

Materials and Methods.

Preparation of collagen gels.

Collagen gels were prepared according to manufacture's instructions (Gibco, Invitrogen). Briefly, a solution of type I collagen from rat tail, at the concentration of 2mg/ml, was mixed with DMEM 5X (Gibco) and 0.025 N NaOH and allowed to polymerize at 37°C for 30 minutes (Fig. 1). This basal condition is referred as condition #A. Moreover, gels, during the preparation steps already described, were supplemented with: 2,5 mM β-glycerophosphate (β-GP, Sigma-Aldrich) and 1,25 mM $CaCl_2$ (BDH AnalR) as potential sources of P and Ca ions necessary to form hydroxyapatite (condition #B); β-GP, $CaCl_2$ as in condition #B, but in the presence of 1U/ml Alkaline Phosphatase from bovine intestinal mucosa (ALP, Sigma-Aldrich), since this enzyme is required to cleave P from substrates (condition #C). Finally, to the complete mixture, as in condition #C, different concentrations of $MgCl_2$ (Riedel-DE Haen) were added (condition #D). As a result of a set of preliminary experiments, a working solution of 62,5 mM $MgCl_2$ was used.

400 μl of each collagenous mix were quickly put in a 24-well-plate and, after polymerization, gels were gently detached before adding 500 μl of DMEM. Where required, human dermal fibroblasts, at the concentration of 1.2×10^5 cells/ml, were added to collagen solutions before gel polymerization. In order to allow cells to settle in the 3D structure, gels were let to stabilize for 24h before morphological analyses.

Environmental Scanning and Scanning Transmission Electron Microscopy (ESEM and SEM-STEM) and Energy-Dispersive Spectroscopy (EDS)

After dehydration and air drying, without any further treatment, samples were observed by environmental scanning electron microscopy (FEI-ESEM Quanta 200) (FEI, Hillsboro, OR, USA). Accelerating voltage was 5 kV for morphological observations and 20 kV for micro-analytical analyses. A solid state detector (SSD) for backscattered electrons and a large field detector (LFD) for secondary electrons were used for imaging. Microanalysis was performed using X-EDS (Oxford

– INCA-350) (Oxford Instruments, Austin, TX, USA). Collagen fibril diameters were evaluated using the Measure and Label plugin for ImageJ. For each experimental condition, at least 30 collagen fibrils from two different areas of the gel (magnification = 10000×) were measured.
For STEM observations, collagen gels were routinely fixed in 2.5% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Spurr resin. Ultrathin sections stained with uranyl acetate and lead citrate were observed by SEM-STEM (Nova NanoSEM 450-STEM, FEI).

Histochemistry and light microscopy

Collagen gels were fixed with 10% neutral buffered formalin, dehydrated and paraffin embedded. Sections, 4-µm thick, were obtained with a LEICA microtome and immobilized on glass slides. After deparaffinization, samples were stained with Anilin blue and observed by light microscopy (Zeiss). Von Kossa staining was applied to detected mineralization [29,30]. Briefly, sections were placed in demineralized water and exposed to 5% silver nitrate solution for 30 min under UV irradiation. After rinsing in demineralized water, sections were exposed to 2% sodium thiosulfate for 5 min, rinsed in demineralized water, dehydrated and covered with glass coverslips for microscopic analyses.

Gel retraction and live/dead assay

Gels were prepared as described above. Human dermal fibroblasts (HDF), purchased from ThermoFisher Scientific (Waltham, MA, USA), were routinely grown in DMEM supplemented with 10% foetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific) according to standard procedures [41]. HDF were added to gels at a final seeding density of 1.2×10^5 cells/ml in 2mg/ml of collagen. In gel retraction experiments, cell-seeded collagen gels were prepared in duplicate in 24-well plates and evaluated at different time points (0, 6, 24 and 48 h). After 30 min at 37 °C, polymerized gels were immersed in 500 µl of standard culture medium (DMEM supplemented with 10% FBS) and detached from the wells using a small spatula. Addition of serum is a fundamental requirement, since in its absence cells are not able to retract [32,33]. Three independent experiments were carried out and free-floating gel retraction was assessed at the indicated time points as a percentage of the initial gel area.

Cell viability was assessed by the "live/dead staining" using calcein AM (Sigma) and propidium iodide (Sigma). Collagen gels were rinsed in PBS, incubated with 1 ml of calcein-AM (1 mg/mL in PBS) and of propidium iodide (1 mg/mL in PBS). Cells were incubated for 10 min at room

temperature, washed twice with PBS, and observed by fluorescence microscopy at 24 and 48 h after seeding.

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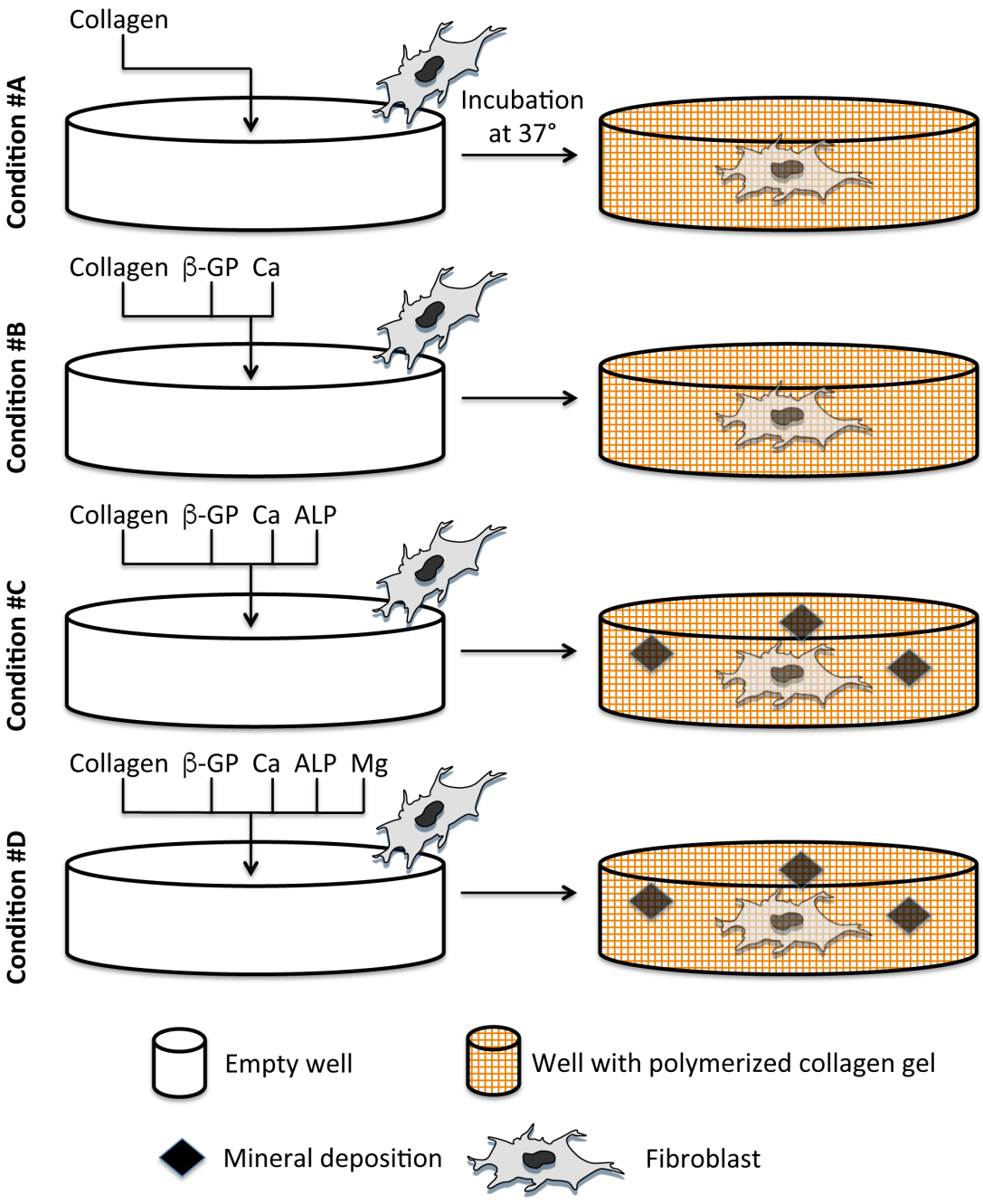


Fig. 1. Collagen gels were prepared providing four different experimental conditions. Condition #A is represented by a standard collagen gel and, in sequence, the following components were added: β-glycerophosphate (β-GP) and $CaCl_2$ (Ca) (condition #B), Alkaline Phosphatase (ALP) (condition #C) and $MgCl_2$ (Mg) (condition #D). Presence of ALP in #C and #D promotes the cleavage β-GP to release phosphate that can react with Ca ions to form insoluble calcium phosphate.

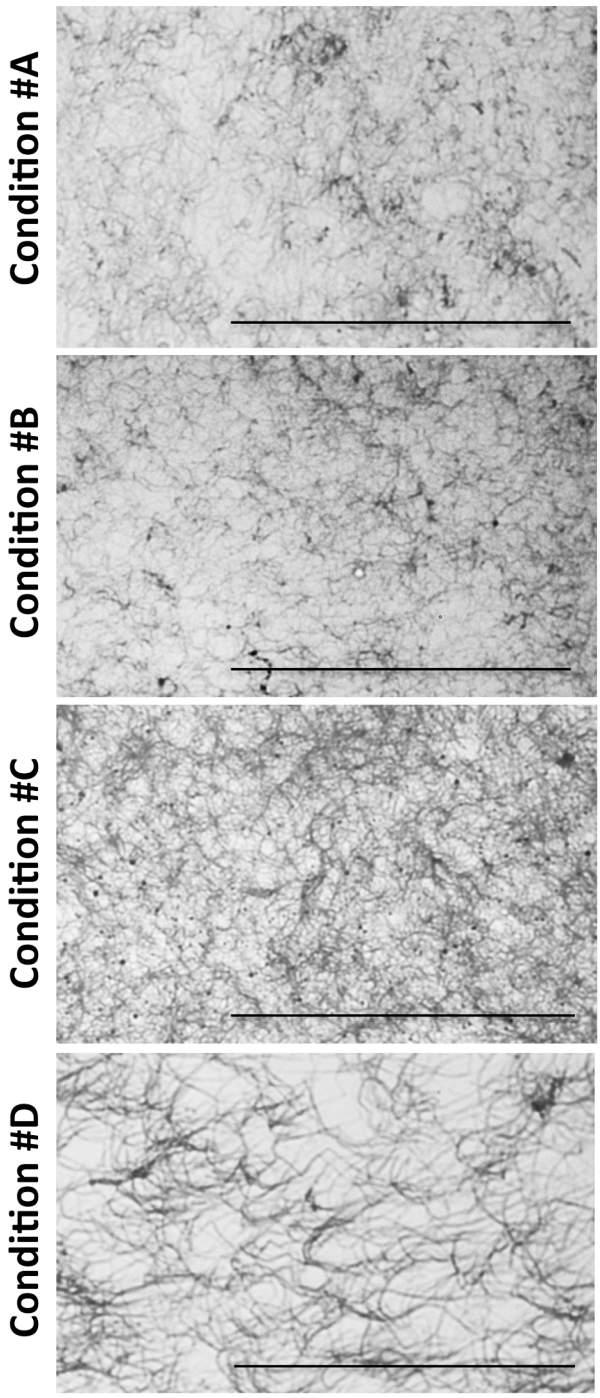


Fig. 2. The fibrillar organization of collagen gels is investigated by light microscopy. Images correspond to the four experimental conditions as in Fig 1. Bar: 100 μm

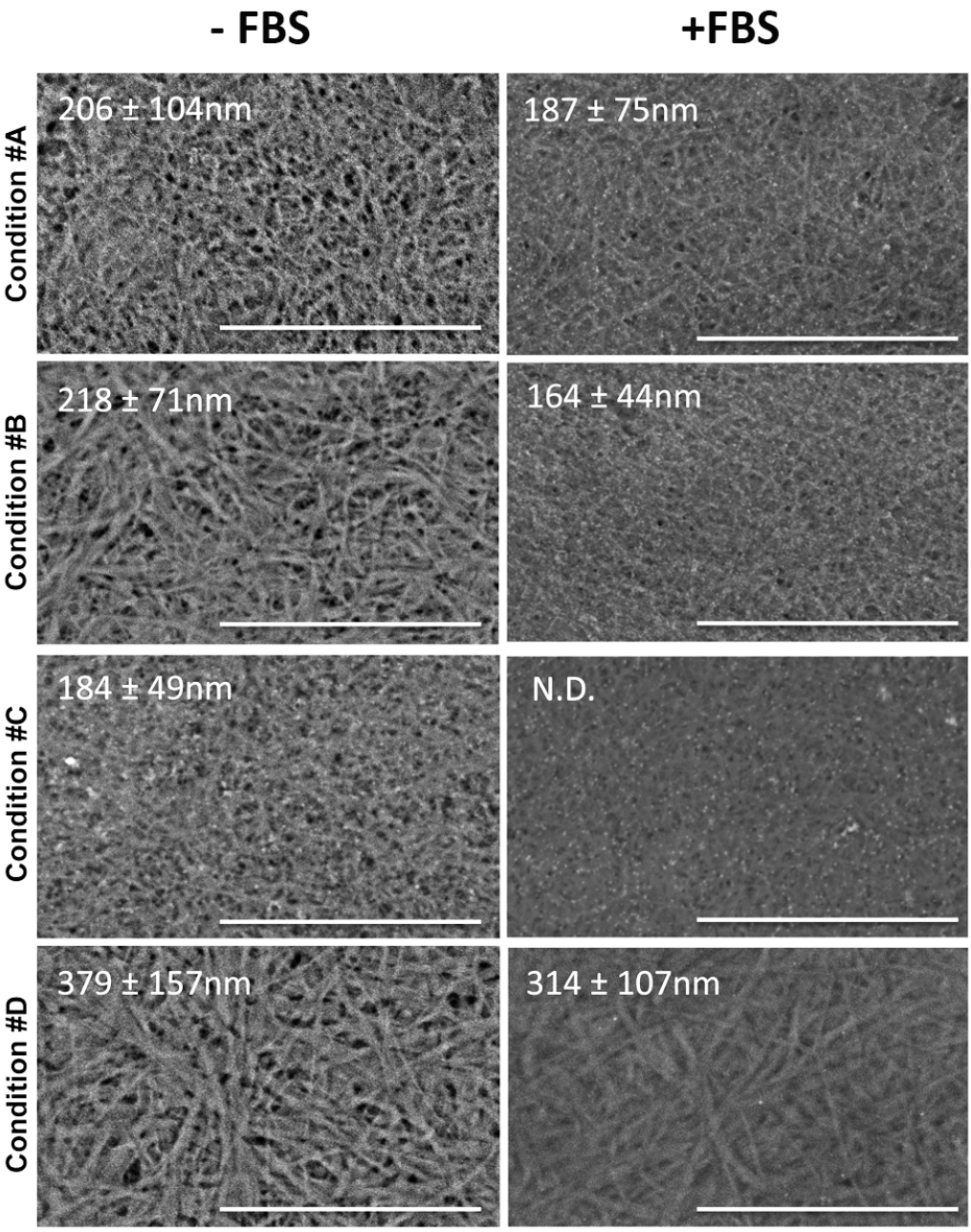


Fig. 3. Collagen gel organization, in absence/presence of FBS, is investigated by environmental scanning electron microscopy (ESEM). Images correspond to the four experimental conditions as in Fig. 1. Values represent the mean diameter of collagen fibrils ±SD. Bar: 100 μm

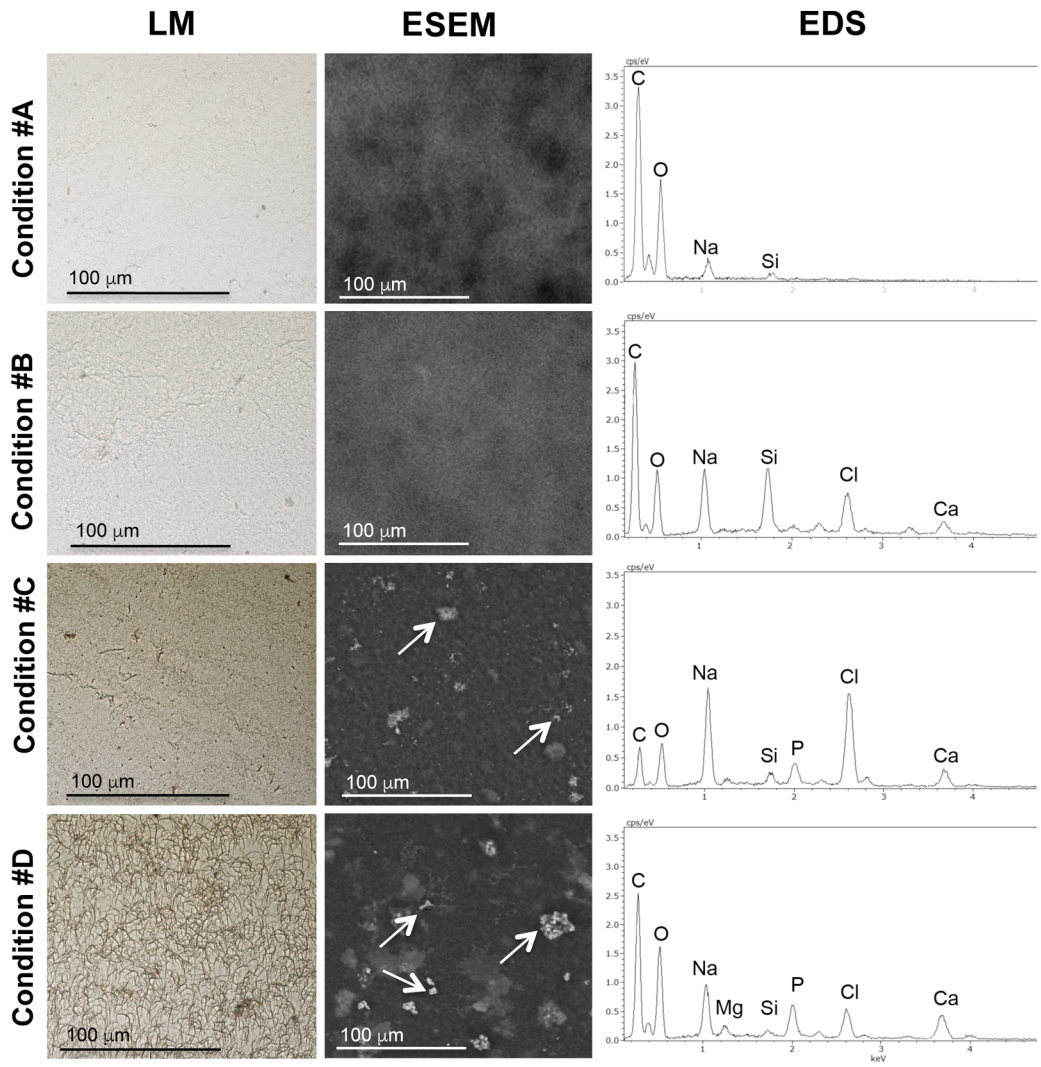


Fig. 4. The presence of mineral deposits is visualized by von Kossa staining (brown) with Light Microscopy (LM). Shape and composition of mineral deposits is shown by Environmental Scanning Electron Microscopy (ESEM) and Energy-Dispersive Spectroscopy spectra (EDS).

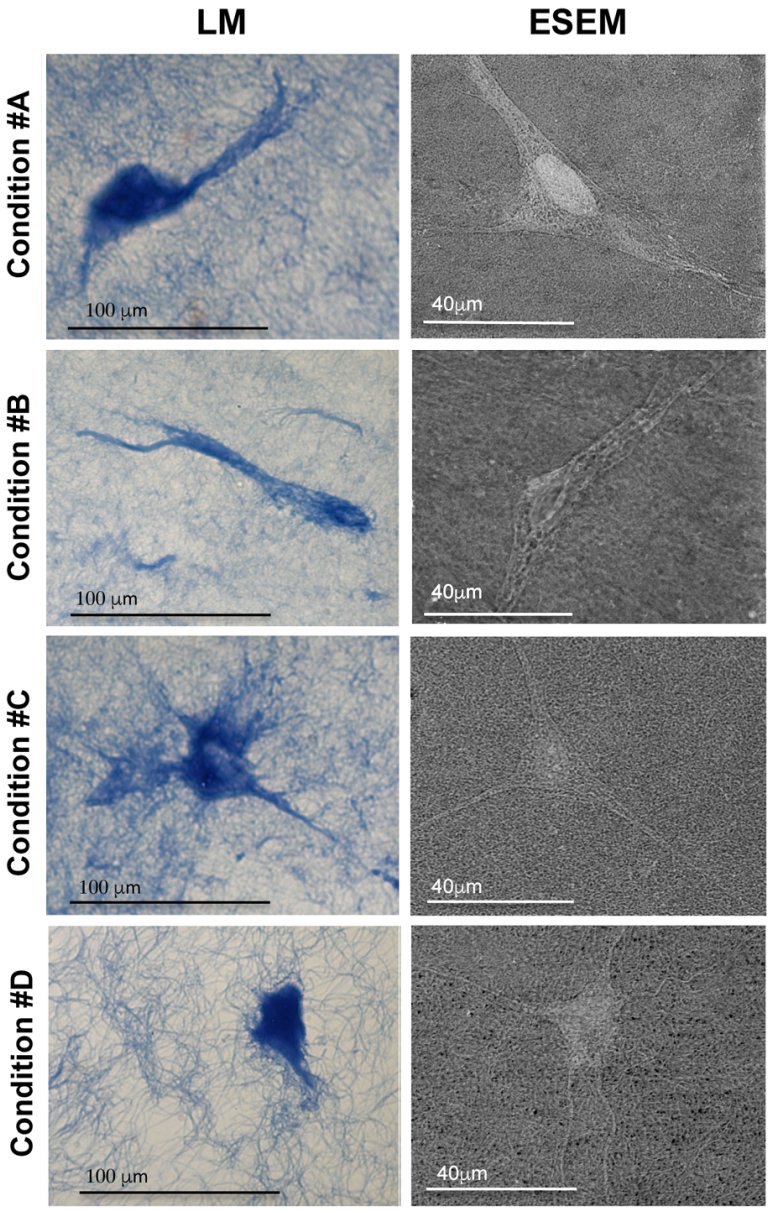


Fig. 5. Morphology of fibroblasts embedded within collagen gels in the four experimental conditions as in Fig. 1. Cells were observed by light (LM) and by Environmental Scanning Electron Microscopy (ESEM). In condition #D cells have a less elongated shape.

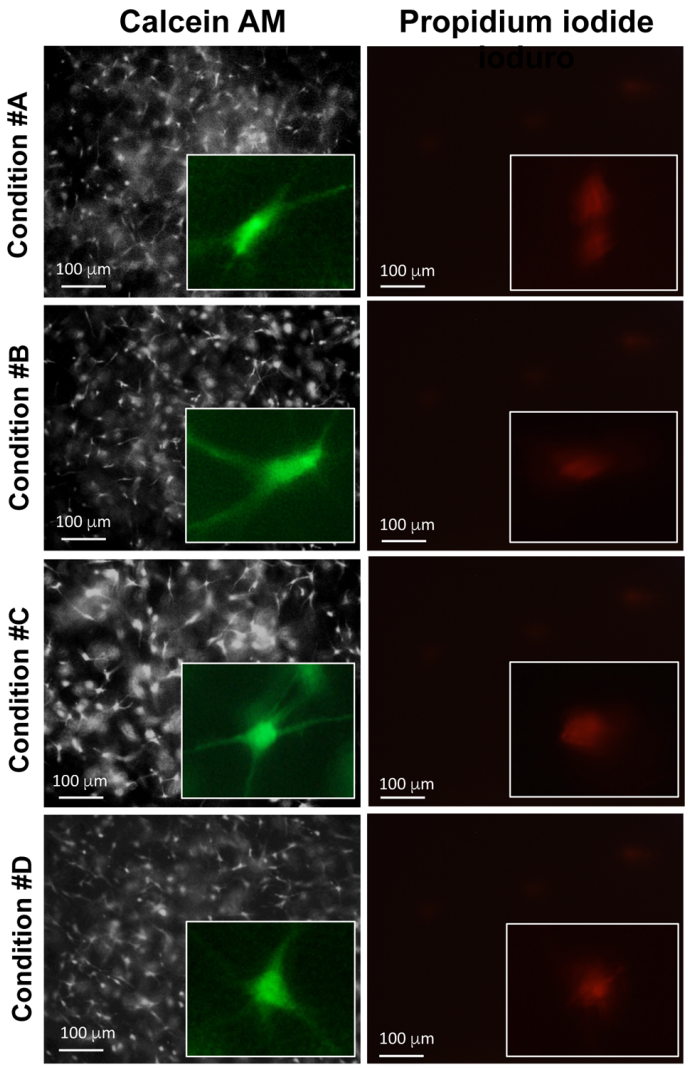
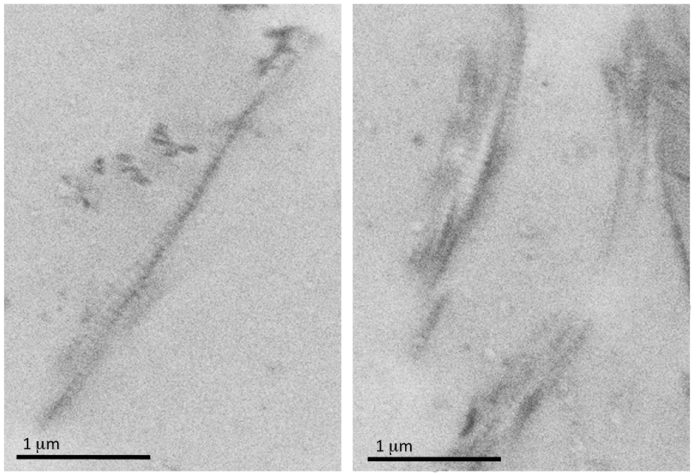
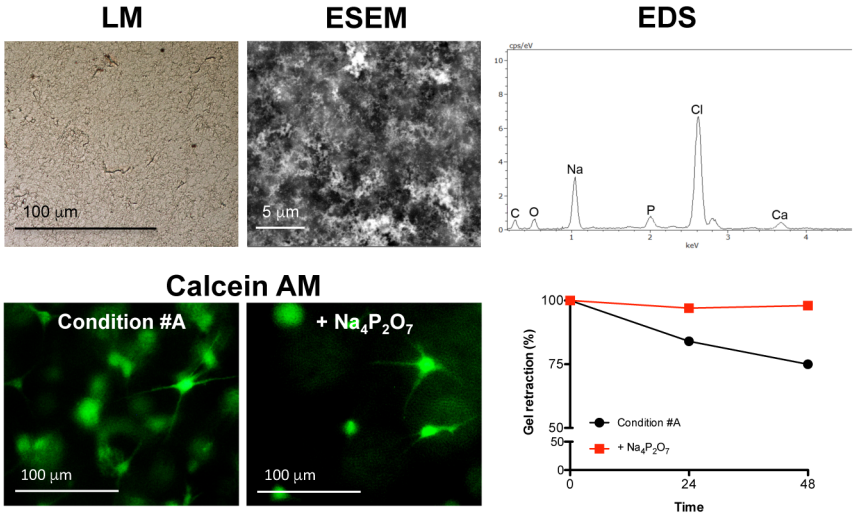


Fig. 6. Live/dead fluorescence microscopy of fibroblasts embedded within collagen gels. Experimental conditions are those illustrated in Fig. 1. Cell viability was assessed by calcein AM staining and fluorescence was visible in all condition. Only few dead cells, stained with propidium iodide, were seen in all samples.



Suppl. Fig. 1. Scanning Transmission Electron Microscopy (SEM-STEM) of fibrils forming collagen gels in two different experimental conditions (#B, left and #D, right).



Suppl. Fig. 2. Mineralization of collagen gel was obtained with addition of $CaCl_2$ e of $Na_4P_2O_7$ (3mM). The presence of mineral deposits is visualized by von Kossa staining (brown) with Light Microscopy (LM). Shape and composition of mineral deposits is shown by Environmental Scanning Electron Microscopy (ESEM) and Energy-Dispersive Spectroscopy spectra (EDS). Cell viability was assessed by calcein AM staining and collagen retraction is shown in graphs.

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