

1 **Fibroblasts from patients affected by Pseudoxanthoma elasticum exhibit an**
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3 **altered PPi metabolism and are more responsive to pro-calcifying stimuli.**
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1 **Abbreviation list**
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3 ALPL/TNAP (gene/protein), tissue-nonspecific alkaline phosphatase isozyme
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5 ANKH/ANKH (gene/protein), progressive ankylosis protein homolog
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7 CM, calcifying medium
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9 ENPPI/PC1 (gene/protein), ectonucleotide pyrophosphatase/phosphodiesterase family
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11 member 1
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13 MGP, Matrix Gla Protein
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15 Pi, inorganic phosphate
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17 PPi, inorganic pyrophosphate
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19 PXE, Pseudoxanthoma elasticum
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21 SPP1/OPN (gene/protein), osteopontin
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1 **Abstract**

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3 **Background.** Pseudoxanthoma elasticum (PXE) is a genetic disorder characterized by
4 progressive calcification of soft connective tissues. The pathogenesis is still hard to pin down.
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6 In PXE dermal fibroblasts, in addition to impaired carboxylation of the vitamin K-dependent
7 inhibitor matrix Gla protein (MGP), we have also demonstrated an up-regulation of alkaline
8 phosphatase activity. In the light of these data we have suggested that both calcium and
9 phosphate metabolism might be locally altered, both pathways acting in synergy on the
10 occurrence of matrix calcification.

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12 **Objective.** This study aims to better explore if cultured PXE fibroblasts, compared to
13 control cells, exhibit a modified inorganic pyrophosphate (PPi) metabolism and are more
14 responsive to pro-calcifying stimuli.
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16 **Methods:** Primary human dermal fibroblasts isolated from healthy individuals and from
17 PXE patients were cultured for different time points in standard and in pro-calcifying
18 media. The expression of ANKH/ANKH, ENPPI/PC1, ALPL/TNAP, SPPI/OPN were
19 evaluated by PCR and Western blot, respectively. TNAP activity was measured by
20 spectrophotometric analyses, whereas calcification was investigated by light and electron
21 microscopy as well as by micro-analytical techniques.
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23 **Results.** In the presence of pro-calcifying stimuli, dermal fibroblasts alter their phenotype
24 favouring matrix mineralization. In particular, ENPPI/PC1 and SPPI/OPN expression, as
25 well as TNAP activity, were differently expressed in control and in PXE fibroblasts.
26 Moreover, in pathologic cells the ratio between factors favouring and reducing PPi
27 availability exhibit a more pronounced shift towards a pro-calcifying balance.
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29 **Conclusion.** PXE fibroblasts are more susceptible to pro-calcifying stimuli and in these
30 cells an altered PPi metabolism contributes to matrix calcification.
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1 **Introduction**

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3 Ectopic calcification is a deleterious and worldwide complication affecting soft
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5 connective tissues [1]. In the last decade, the involvement of numerous genes/proteins
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7 has been disclosed in a number of investigations, indicating that pathologic
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9 mineralization is the result of an active and dynamic balance of pro- and anti-calcifying
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11 mechanisms [2,3]. Never the less, there are no therapeutic options capable to efficiently
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13 inhibit the occurrence of ectopic calcification [4], suggesting that at least some of the
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15 pathogenetic mechanisms are still elusive and need to be further explored.
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19 Within this context, Pseudoxanthoma elasticum (PXE), an autosomal recessive disorder
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21 mainly affecting skin, eyes and the cardiovascular system, is typically characterized by
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23 a progressive mineralization of the elastic component of soft connective tissues [5]
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25 without any clear evidence of inflammation, cell necrosis or apoptosis [6]. Moreover, in
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27 PXE, in the absence of increased circulating levels of calcium and phosphate [7],
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29 calcified elastic fibres have a calcium/phosphate ratio similar to that of physiologically
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31 mineralized tissues as bone [8]. Surprisingly, PXE is associated to mutations in the
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33 ABCC6 gene [9] that encodes for a transmembrane protein highly expressed in liver and
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35 kidney, whereas it is barely detectable in soft connective tissues [6]. Clinical data and
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37 experimental observations indicate that, in PXE, ectopic calcification is not a passively
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39 diffused phenomenon, but affects peculiar matrix components in specific anatomical
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41 areas, although the pathogenetic mechanisms are still elusive. It has been suggested that
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43 elastic fibre calcification is the result of altered regulatory mechanisms that, possibly
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45 mediated or induced by factors abnormally released from the liver [6, 10-12], may
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47 influence mesenchymal cells. These cells are responsible for triggering soft connective
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49 tissue mineralization in defined areas and not in the whole matrix, as it would occur
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1 simply due to the presence of altered circulating levels of calcium and phosphate and/or
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3 of other systemic factors [13-15]. At least in the skin, fibroblasts should be considered
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5 the principal candidates for the occurrence and progression of dermal ectopic
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7 calcification [16].
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10 In previous *in vitro* studies, we have demonstrated that PXE fibroblasts are unable to
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12 properly carboxylate matrix Gla protein (MGP) [7,17-18], a potent vitamin K-dependent
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14 inhibitor of calcium deposition [19] and are characterized by increased alkaline
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16 phosphatase activity [7]. However, up-regulated TNAP activity is necessary, but not
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18 sufficient, to induce the mineralization process, as demonstrated in *in vitro* standard cell
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20 culture conditions [18]. An appropriate ratio between inducers and inhibitors of
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22 calcifications (either present in serum supplemented media or produced by cells) is in
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24 fact required to have hydroxyapatite actively deposited on the cellular monolayer [7, 20].
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28 Aim of this study was to investigate whether primary human dermal fibroblasts from
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30 PXE patients are more responsive to pro-calcifying stimuli than control cells from
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32 healthy individuals. Moreover, we have investigated if these fibroblasts exhibit a
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34 differential expression of genes/proteins involved in the inorganic pyrophosphate (PPi)
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36 metabolism (i.e. progressive ankylosis protein homolog, *ANKH/ANKH*; ectonucleotide
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38 pyrophosphatase/phosphodiesterase family member 1, *ENPPI/PC1*; tissue-non specific
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40 alkaline phosphatase isozyme, *ALPL/TNAP*). Moreover, given the regulatory role of
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42 PC1, *ANKH* and *TNAP* on osteopontin (*SPP1/OPN*) [21], the expression of this
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44 inhibitor of mineral crystal growth [22-23] was also investigated.
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52 To address these questions, cells were cultured in standard (DMEM) and in calcifying
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54 media (CM) (i.e. in the absence/presence of calcifying stimuli, respectively) and
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56 evaluated at early and late time points (8 hours and 20 days, respectively). These two
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1 time points were selected with the aim to investigate i) if changes occurring in cells well
2 before mineral deposition takes place could be indicative of a particular susceptibility of
3 cells to pro-calcifying stimuli, and ii) if the mineralizing environment provides a source
4 of signalling cues that may further influence cell phenotype.
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10 **Materials and Methods**

11 **Cells**

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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, New York, 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).

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 75cm² flasks (Nunc,
Roskilde, Denmark) with DMEM supplemented with 10% FBS, penicillin 100U/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)

1 (DMEM) or in a more complex calcifying medium (CM) (i.e. DMEM supplemented
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3 with 10mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 10nM dexamethasone
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5 (Sigma-Aldrich, Milan, Italy) [7]. Media were replaced three times a week.
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11 ***In vitro* calcification: von Kossa staining and analysis by Environmental Scanning**
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13 **Electron Microscopy (ESEM) and X-ray Energy Dispersive Spectroscopy (EDS).**

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15 Fibroblasts were routinely cultured in six-well plates (BD-Falcon, Franklin Lakes, NJ)
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17 up to confluence, when CM replaced the standard medium. At different time points,
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19 cells were fixed in 4% paraformaldehyde, stained with 2.5% silver nitrate, placed under
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21 a UV lamp for 30 min and rinsed with distilled water before treatment with 5% sodium
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23 thiosulfate for two minutes. Von Kossa-positive (black) deposits were observed after
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25 alcohol washes. Mineralized areas were quantified using the Image J software.
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27 Experiments were conducted two times on all cell lines. At the same time, parallel
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29 cultures were fixed in methanol for 10 min and then samples were observed with a
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31 scanning electron microscope (FEI-ESEM Quanta 200) (FEI, Hillsboro, OR, USA) in
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33 Low Vacuum mode. Solid State Detector (SSD) for backscattered electrons and Large
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35 Field Detector (LFD) for secondary electrons were used for imaging. Microanalysis was
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37 performed using X-EDS (Oxford - INCA-350)(Oxford Instruments, Austin, TX, USA)
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50 **Tissue non specific alkaline phosphatase isozyme activity**

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52 Fibroblasts were detached by enzymatic treatment and washed three times with PBS.
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54 Cells were lysed by appropriate buffer containing 0.1% Triton X-100 (Sigma). Cell
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56 lysate (20 μ L) was mixed with 100 μ L Tris-glycine buffer pH 10.3 (50mM Tris-HCl,
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1 100mM glycine and 2mM MgCl₂) and 100 μL of p-nitrophenyl phosphate (Sigma). The
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3 reaction mix was incubated at 37°C for 30 min and the reaction stopped by adding 50μl
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5 of 3M NaOH. Absorbance was measured at 405 nm in a microplate reader. Enzymatic
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7 activity was normalized to cell number. Control samples were set at 1. Experiments
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9 were performed at least three times in triplicate.
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16 **RNA preparation and quantitative real-time PCR (qRT-PCR).**

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18 Confluent cells were cultured in DMEM or in CM for a short (8 hours, i.e. early CM)
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20 and a longer length of time (20 days, i.e. late CM). At each time point, total cellular
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22 RNA was extracted using RNeasy Protect cells Mini kit reagent (Qiagen, Valencia,
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24 CA) following manufacturer's instructions. Quality and quantity of RNA were checked
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26 with a spectrophotometer and by agarose gel electrophoresis. cDNA was then reverse-
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28 transcribed from 3 μg of total RNA using superscript III Reverse Transcriptase
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30 (Invitrogen, Monza, Italy). The amplification efficiency of primers was between 95%
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32 and 105% [25]. Primers and their characteristics are listed in Table 1. A 3x diluted
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34 cDNA sample was further amplified on a iCycler (BioRad, Segrate, Italy) using SYBR[®]
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36 GreenER[™]qPCR SuperMix (Invitrogen), according to manufacturer's instructions.
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38 Thermal cycling parameters were set at 95°C for 5 min, 40 cycles at 95°C for 15 sec, an
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40 annealing temperature of 60°C for 30 sec, and of 72°C for 15 sec, followed by melting
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42 curve analysis with a temperature ranging from 95°C to 55°C.
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50 Gene expression in each sample was normalized to the expression of a housekeeping
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52 gene (*CLK2*) that has been considered more suitable for determinations in dermal
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54 fibroblasts [26]. Data were evaluated using the Pfaffl method [27] based on real-time
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1 PCR efficiencies and compared with control cells. All qRT-PCR analyses were carried
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4 out in triplicate.
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8 **Protein extraction and Western blot (WB)** 9

10 Cells were homogenized in RIPA buffer with protease inhibitors (Sigma), centrifuged
11 and supernatants collected and stored at -80°C until analysis. Protein concentration was
12 and supernatants collected and stored at -80°C until analysis. Protein concentration was
13 measured in each sample according to Bradford assay [28] in order to load equal
14 amounts of proteins. After separation by 1D PAGE [18], proteins were transferred to
15 nitrocellulose and incubated with primary antibodies and thereafter with appropriate
16 horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge,
17 UK). The following primary antibodies against ANKH (Abcam), OPN (Abcam), PC1
18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNAP (Santa Cruz Biotechnology),
19 β actin (Sigma) and S100A4 (Abcam) were used. Experiments were performed at least
20 two times with all different cell lines.
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37 **Statistical analysis** 38

39 Values were analysed by Mann Whitney and by Anova test with significance at $p < 0.05$.
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42 Statistical data were obtained using GraphPad software 5.0 (San Diego, CA, USA).
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47 **Results** 48

49 In order to induce mineralization *in vitro*, fibroblasts, as all other mesenchymal cells,
50 must be cultured in a pro-calcifying medium that provides cells of the appropriate
51 environment [20].
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1 Mineral deposits were characterized by environmental scanning electron microscopy
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3 (ESEM) and by micro-analytical analyses. By ESEM, calcification appeared as small
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5 round precipitates that, with time in culture, coalesced into bigger areas of
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7 mineralization (figure 1A, C, D). However, even after 40 days of culture in CM, mineral
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9 deposits were not spread all over the cellular monolayer, but there were areas in which
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11 cells were devoid of mineral precipitates (figure 1C, D). EDS spectra (figure 1B)
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13 demonstrated that calcified areas were made of calcium and phosphate, whose
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15 concentration was proportional to the presence and to the size of precipitates (figure 1
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17 B), thus confirming that, although in an *in vitro* experimental model, the mineralization
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19 process mimic what takes place *in vivo* [8]. Comparable data were obtained on cell
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21 cultures from PXE patients (figure 1) and from control individuals (data not shown).

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23 The extent of calcification was evaluated in control and PXE fibroblasts by light
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25 microscopy after the von Kossa staining (figure 2). As already demonstrated [7], cells
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27 cultured in DMEM never exhibited mineral deposition that, on the contrary, occurred in
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29 the presence of CM starting around day 20 and then progressively increased (figure 2).
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31 Crystal deposition was rather heterogeneous on the cellular monolayer with a variability
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33 being highly dependent on cell strain. Consistently, some PXE fibroblast cell strains
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35 exhibited a more extended surface area covered by mineral precipitates compared to
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37 control cells (figure 2).

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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 analysed by Western blot on fibroblasts cultured in DMEM and in CM at early and late time points (8 hours and 20 days, respectively). Both actin and S100A4 were expressed in all culture conditions and in all cell strains. Interestingly,

1 their expression significantly decreased after several days in CM (figure 3),
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3 demonstrating that these proteins were down-regulated when cells were actively
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5 involved in matrix calcification and that fibroblasts modified their phenotype in a
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7 mineralized environment.
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11 Since we have demonstrated that TNAP activity is up-regulated in PXE, it was
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13 suggested that phosphate metabolism may be locally altered, thus contributing to the
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15 mineralization process [7]. Pyrophosphate-related gene and protein expressions were
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17 therefore investigated on control and PXE fibroblasts. Cells were cultured in a pro-
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19 calcifying environment in order to disclose, in an *in-vitro* system, whether PXE
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21 compared to control fibroblasts were more responsive to mineralization stimuli.
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25 No significant differences in the expression of *ANKH/ANKH*, *ENPPI/PC1*,
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27 *ALPL/TNAP* were detected at mRNA and protein levels in all cell strains cultured in
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29 DMEM (data not shown), in agreement with the inability of cells to calcify in these *in*
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31 *vitro* experimental conditions. By contrast, control fibroblasts cultured in CM at early
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33 and late time points revealed that, compared to basal levels of expression (i.e. confluent
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35 cells in DMEM), *ENPPI/PC1* expression was significantly up-regulated at a late time
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37 point, when mineralization takes place (figure 4). Differences in *ANKH*, and *ALPL*
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39 expression were negligible at all time points (figure 4).
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45 A similar trend was observed also in PXE fibroblasts (figure 5), however, compared to
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47 basal levels, PC1 expression was significantly down- and up-regulated at early and late
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49 time points, respectively.
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52 Comparison of control and PXE fibroblasts (figure 6) revealed that PXE cells, at the
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54 early time point in CM, had a significant down-regulation of PC1 expression, whereas
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56 values were similarly up-regulated in all cell strains at later time points (figure 6B).
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1 Differences in ANKH expression were negligible in all experimental conditions (figure
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3 6A). As already suggested [7], the mineralization process, at least *in vitro*, is associated
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5 to a progressive up-regulation of TNAP activity. In agreement with these observations,
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7 in the present study enzyme activity was progressively up-regulated in all cell strains
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9 (figure 6C), but values were significantly higher in PXE compared to control
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11 fibroblasts. Furthermore, since calcification is the results of a ratio between inducers
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13 and inhibitors of the mineralization process, including those regulating PPi, in figure 6D
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15 is shown the ratio between factors involved in PPi production and transport and factors
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17 that utilize PPi as a substrate for Pi release. This ratio, taken as an indicator of the
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19 mineralization potential of cells, appeared to progressively decrease as mineralization
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21 takes place in both cell lines. Interestingly, PXE cells have significantly lower values
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23 than control fibroblasts (figure 6D).

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30 In the extracellular milieu, osteopontin acts as a calcification inhibitor whose expression
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32 can be modulated by phosphate and pyrophosphate availability and by TNAP activity
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34 [21, 31-32]. *SPPI* expression was significantly down-regulated in control and PXE
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36 fibroblasts at early time point in CM (figure 7A, C). Moreover, OPN expression, at
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38 early time point, was significant reduced in PXE compared to control cells (figure 7E),
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40 but it appeared increased up to basal levels in all cell strains at late compared to early
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42 time points (figure 7B, D).

43 44 45 46 47 48 49 **Discussion**

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52 Pseudoxanthoma elasticum (PXE) is a genetic disease characterized by progressive
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54 ectopic calcification in soft connective tissues, including the skin [6]. In these patients,
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56 plasma levels of calcium and phosphate are normal, suggesting that other mechanisms
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1 are involved in the control of mineralization [7]. We have previously shown that, in the
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3 absence of any altered value in the circulation, TNAP activity is up-regulated in PXE
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5 fibroblasts and that levamisole, a specific TNAP inhibitor, suppresses mineralization *in*
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7 *vitro*. Therefore, it was hypothesized that in these cells, in addition to impaired MGP
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9 carboxylation, a modified PPi metabolism contributes to soft connective tissue
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11 calcification [7]. In agreement with this hypothesis, fibroblasts, being mesenchymal
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13 cells responsible for the correct amount and the appropriate quality of cellular and
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15 extracellular proteins, regulate the local balance of mineralization inducers and
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17 inhibitors and therefore can be advantageously used as an *in vitro* experimental model
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23 [16].
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25 The present work aimed to study the effects of a pro-calcifying environment on control
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27 and PXE dermal fibroblasts by specifically looking at changes in the expression of
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29 genes and proteins involved in PPi metabolism. In particular, we have investigated the
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31 expression of PC1 that generates PP_i from nucleoside triphosphates, of ANKH that
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33 mediates intracellular to extracellular PPi transport and of TNAP that hydrolyses PPi
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37 [21].
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40 As already demonstrated, a standard cell culture environment is unsuitable to promote
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42 mineralization *in vitro*, independently from the ability of cells to induce calcification *in*
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44 *vivo* [33]. Consistently, in the present study, even after a long time of culture in DMEM,
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46 fibroblasts maintained their phenotype and mineralization never occurred. Moreover,
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48 the expression of mineralization-related genes/proteins regulating PPi metabolism
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50 exhibited negligible differences at various time points in culture as well as between
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52 control and PXE fibroblasts.
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1 Therefore cells were cultured in an appropriate pro-calcifying environment that
2 provides the stimuli necessary to induce the mineralization process *in vitro* [20, 34]. In
3 order to avoid the passive mineral deposition that, upon inorganic phosphate
4 supplementation, was demonstrated *in vitro*, even without cells [35], a complex medium
5 containing ascorbic acid, β -glycerophosphate and dexamethasone was used [7, 20].
6 When cells were cultured in this pro-calcifying environment, there was a progressive
7 hydroxyapatite deposition, even though mineralization was not homogeneously
8 distributed over the monolayer. Calcification, in fact, is not a passive phenomenon and
9 cells, even in the same environment, are actively and differently responsive to
10 mineralization stimuli [20]. Present findings could explain why, in PXE, calcification
11 occurs in specific areas in the absence of higher circulating levels of calcium and
12 phosphate and without any evidence of inflammation or cell death.
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30 The continuous presence of pro-calcifying stimuli is associated to cellular phenotypic
31 changes [29-30]. In the present study, at later time points, fibroblasts reveal a
32 cytoskeletal reorganization with reduced actin expression. Moreover, S100A4, a
33 calcium-binding protein known to exert an inhibitory role on mineral deposition [29],
34 was decreased to undetectable levels when the mineralized matrix is formed, indicating
35 that calcification comes by changes in fibroblasts' behaviour.
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45 Surprisingly, at early time points, before mineralization and before changes in
46 fibroblasts' phenotype, PXE compared to control cells were characterized by
47 significantly reduced expression of PC1 and increased TNAP activity. These changes
48 act in synergy reducing the availability of PPI, a condition that represents a prerequisite
49 for the mineralization process to take place [36]. Like PPI, OPN is a potent inhibitor of
50 hydroxyapatite crystal growth [22-23]. Moreover, it has been demonstrated in
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1 osteoblasts that PPI is a specific signal for the induction of OPN expression [32] and
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3 that PPI deficiency in *ENPPI*^{-/-} mice results in OPN deficiency [31]. In agreement with
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5 these observations a down-regulation of OPN has been demonstrated in PXE
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7 fibroblasts.
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10 The altered expression of PPI-related proteins, as clearly shown at the early time point,
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12 indicates that PXE compared to control fibroblasts are particularly sensitive to a pro-
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14 calcifying environment.
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17 At late time points, in a mineralizing environment, both control and PXE cells exhibited
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19 a significant up-regulation of *ENPPI/PC1* as an attempt to counteract the mineralization
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21 process. However, PPI is a substrate for TNAP, whose activity was significantly up-
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23 regulated with time in CM, especially in PXE fibroblasts. After 20 days of culture,
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25 compared to the early time point in CM, OPN was up-regulated towards basal levels of
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27 expression.
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32 It has to be noted that at later time points, after several days in the presence of pro-
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34 calcifying stimuli, the different response of control and PXE fibroblasts, observed at
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36 early time points, was no further detectable, being masked as a consequence of a
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38 secondary response of fibroblasts to the mineralized environment and/or of cumulative
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40 effects due to the extended permanence in CM.
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45 We are aware that these culture conditions represent an artificial system, as are all *in*
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47 *vitro* models, since they should mimic in shorter times (few days or weeks) what takes
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49 place *in vivo* along the years under complex regulatory mechanisms active at both local
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51 and systemic levels. Never the less, results demonstrate that *in vitro*, if an adequate
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53 availability of calcifying stimuli are provided to cells, fibroblasts promote mineral
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55 deposition. These events are only partly dependent of TNAP activity, since enzyme up-
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1 regulation *per se* is not sufficient to favour the calcification process, as demonstrated for
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3 instance in cells cultured in standard medium [7], even in the presence of higher levels
4
5 of enzyme activity. Never the less, even in PXE fibroblasts cultured in DMEM, the ratio
6
7 between inducers and inhibitors of PPi metabolism is already shifted towards a pro-
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9 calcifying balance, suggesting that these cells may be more susceptible to
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11 mineralization stimuli compared to control fibroblasts. Mineralization takes place in all
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13 cell strains after several days in CM, when threshold levels of calcification inducers
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15 exceed that of inhibitors. However, PXE fibroblasts, although with a variability
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17 depending on cell strain, showed higher amount of mineralization, supporting the
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19 hypothesis that these cells are more responsive to calcifying stimuli.
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25 Moreover, it has to be underlined that in PXE, both *in vivo* and *in vitro*, the lower
26
27 amount of carboxylated-MGP causes soft connective tissues being devoid of an
28
29 appropriate and effective protection against mineral deposition [7, 17-18]. Therefore, in
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31 PXE, both reduced MGP carboxylation and altered PPi metabolism may synergistically
32
33 contribute to ectopic calcification.
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37 In conclusion, mineralization may depend on fibroblast's subtype behaviour and
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39 responsiveness [37], on matrix composition (both in terms of quality and quantity of
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41 secreted proteins as well as on proteolytic activities) [6, 38-40], on local calcium and
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43 phosphate availability [6] and on the ratio between mineralization inducers and
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45 inhibitors [7, 16]. Results demonstrate that changes in PPi metabolism are present in
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47 PXE fibroblasts. Moreover, the altered balance between factors promoting or reducing
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49 PPi availability is already present in standard culture conditions, thus turning PXE
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51 fibroblasts into cells more responsive to pro-calcifying stimuli compared to controls.
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57 Therefore, we propose that circulating factors abnormally released in the circulation
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1 [10-12] are likely responsible for the development of a pro-calcifying environment that
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3 progressively induce responsive fibroblasts to alter their phenotype and to promote the
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5 mineralization process.
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10 **Acknowledgments**

11
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13
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15
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17
18 Modena and Reggio Emilia).
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25 **Conflict of Interests**

26
27 Authors declare no conflict of interests.
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1 **References**

- 2
- 3
- 4 [1] Murshed M, McKee MD: Molecular determinants of extracellular matrix
- 5
- 6 mineralization in bone and blood vessels. *Curr Opin Nephrol Hypertens* 2010;19:
- 7
- 8 359-365.
- 9
- 10
- 11 [2] Giachelli CM: Ectopic calcification: gathering hard facts about soft tissue
- 12
- 13 mineralization. *Am J Pathol* 1999;154:671-675.
- 14
- 15
- 16 [3] Golub EE: Biomineralization and matrix vesicles in biology and pathology. *Semin*
- 17
- 18 *Immunopathol* 2011;33:409-417.
- 19
- 20
- 21 [4] O'Neill WC, Lomashvili KA: Recent progress in the treatment of vascular
- 22
- 23 calcification. *Kidney Int* 2010;78:1232-1239.
- 24
- 25
- 26 [5] Gheduzzi D, Sammarco R, Quaglino D, Bercovitch L, Terry S, Taylor W, et al.:
- 27
- 28 Extracutaneous ultrastructural alterations in pseudoxanthoma elasticum.
- 29
- 30 *Ultrastruct Pathol* 2003;27:375-384.
- 31
- 32
- 33 [6] Quaglino D, Boraldi F, Annovi G, Ronchetti I: The Multifaceted Complexity of
- 34
- 35 Genetic Diseases. A Lesson from Pseudoxanthoma Elasticum. In *Advances in the*
- 36
- 37 *study of genetic disorders*. Edited by K. Ikehara. InTech, Rijeka. 2011, pp.289-
- 38
- 39 318.
- 40
- 41
- 42
- 43 [7] Boraldi F, Annovi G, Vermeer C, Schurgers LJ, Trenti T, Tiozzo R, et al.: Matrix
- 44
- 45 Gla Protein and Alkaline Phosphatase Are Differently Modulated in Human
- 46
- 47 Dermal Fibroblasts from PXE Patients and Controls. *J Invest Dermatol*
- 48
- 49 2013;133:946-954.
- 50
- 51
- 52 [8] Boraldi F, Tonelli M, Gheduzzi D, Pasquali-Ronchetti I, Quaglino D:
- 53
- 54 Identification of mineralized elastic fibers on wet samples by SEM. *Microsc Res*
- 55
- 56 *Tech* 2005;7:296-299.
- 57
- 58
- 59
- 60
- 61
- 62
- 63
- 64
- 65

- 1 [9] Le-Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, et al.:
2
3 Mutations in a gene encoding an ABC transporter cause pseudoxanthoma
4
5 elasticum. *Nat Genet* 2000;25:223-227.
6
7
8 [10] Le Saux O, Bunda S, VanWart CM, Douet V, Got L, Martin L, et al.: Serum
9
10 factors from pseudoxanthoma elasticum patients alter elastic fiber formation in
11
12 vitro. *J Invest Dermatol* 2006;126:1497-1505.
13
14
15 [11] Uitto J, Pulkkinen L, Ringpfeil F: Molecular genetics of pseudoxanthoma
16
17 elasticum: a metabolic disorder at the environment-genome interface? *Trends*
18
19 *Mol Med* 2001;7:13-17.
20
21
22 [12] Jiang Q, Endo M, Dibra F, Wang K, Uitto J: Pseudoxanthoma elasticum is a
23
24 metabolic disease. *J Invest Dermatol* 2009;129:348-354.
25
26
27 [13] Toussaint ND : Extracellular matrix calcification in chronic kidney disease. *Curr*
28
29 *Opin Nephrol Hypertens* 2011;20:360-368.
30
31
32 [14] Herrmann M, Kinkeldey A, Jahnen-Dechent W: Fetuin-A function in systemic
33
34 mineral metabolism. *Trends Cardiovasc Med* 2012;22:197-201.
35
36
37 [15] Schlieper G, Westenfeld R, Brandenburg V, Ketteler M: Inhibitors of
38
39 calcification in blood and urine. *Semin Dial* 2007;20:113-121.
40
41
42 [16] Ronchetti I, Boraldi F, Annovi G, Cianciulli P, Quaglino D: Fibroblast
43
44 involvement in soft connective tissue calcification. *Front Genet* 2013;4:22.
45
46
47 [17] Gheduzzi D, Boraldi F, Annovi G, Paolinelli-DeVincenzi C, Schurgers LJ,
48
49 Vermeer C, et al.: Matrix Gla protein is involved in elastic fiber calcification in
50
51 the dermis of pseudoxanthoma elasticum patients. *Lab Invest* 2007;87:998-1008.
52
53
54 [18] Boraldi F, Annovi G, Guerra D, Paolinelli-DeVincenzi C, Garcia-Fernandez MI,
55
56 Panico F, et al.: Fibroblast protein profile analysis highlights the role of
57
58
59
60
61
62
63
64
65

1 oxidative stress and vitamin K recycling in the pathogenesis of pseudoxanthoma
2 elasticum, *Proteomics – Clin Appl* 2009;3:1084-1098.

3
4
5
6 [19] Schurgers LJ, Cranenburg EC, Vermeer C: Matrix Gla-protein: the calcification
7 inhibitor in need of vitamin K. *Thromb Haemost* 2008;100:593-603.

8
9
10 [20] Buranasinsup S, Sila-Asna M, Bunyarativej N, Bunyaratvej A: In vitro
11 osteogenesis from human-skin-derived precursors cells. *Dev Growth Differ* 2006;
12 48:263–269.

13
14
15
16
17 [21] Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millán JL:
18 Concerted regulation of inorganic pyrophosphate and osteopontin by *akp2*, *enpp1*,
19 and *ank*: an integrated model of the pathogenesis of mineralization disorders. *Am*
20 *J Pathol* 2004;164:1199-1209.

21
22
23
24
25 [22] Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW:
26 Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite
27 formation and growth in a gelatin-gel. *Bone Miner* 1993;22:147-159.

28
29
30
31 [23] Wada T, McKee MD, Steitz S, Giachelli CM: Calcification of vascular smooth
32 muscle cell cultures: Inhibition by osteopontin. *Circ Res* 1999;84:166–178.

33
34
35 [24] Quaglino D, Boraldi F, Barbieri D, Croce A, Tiozzo R, Pasquali-Ronchetti I:
36 Abnormal phenotype of in vitro dermal fibroblasts from patients with
37 Pseudoxanthoma elasticum (PXE). *Biochim Biophys Acta* 2000;1501:51-62.

38
39
40 [25] Klatte M, Bauer P: Accurate Real-time Reverse Transcription quantitative PCR.
41 In Plant Signal Transduction. Edited by T. Pfannschmidt. Humana Press, 2009,
42 pp. 61-77.

- 1 [26] deBrouwer AP, van Bokhoven H, Kremer H: Comparison of 12 reference genes
2 for normalization of gene expression levels in Epstein-Barr virus-transformed
3 lymphoblastoid cell lines and fibroblasts. *Mol Diagn Ther* 2006;10:197-204.
4
5
6
7
8 [27] Pfaffl MW: A new mathematical model for relative quantification in real-time
9 RT-PCR. *Nucleic Acids Res* 2001;29:e45.
10
11
12
13 [28] Bradford MM: A rapid and sensitive method for the quantitation of microgram
14 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*
15 1976;72:248-254.
16
17
18
19
20 [29] Duarte WR, Shibata T, Takenaga K, Takahashi E, Kubota K, Ohya K, et al.:
21 S100A4: a novel negative regulator of mineralization and osteoblast
22 differentiation. *J Bone Miner Res* 2003;18:493-501.
23
24
25
26
27 [30] Rodríguez JP, González M, Ríos S, Cambiazo V: Cytoskeletal organization of
28 human mesenchymal stem cells (MSC) changes during their osteogenic
29 differentiation. *J Cell Biochem* 2004;93:721-731.
30
31
32
33
34 [31] Johnson K, Goding J, van Etten D, Sali A, Hu SI, Farley D, et al.: Linked
35 deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification
36 associated with defective PC-1 and ANK expression. *J Bone Miner Res*
37 2003;18:994–1004.
38
39
40
41
42 [32] Addison WN, Azari F, Sørensen ES, Kaartinen MT, McKee MD: Pyrophosphate
43 inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating
44 osteopontin, and inhibiting alkaline phosphatase activity. *J Biol Chem* 2007;282:
45 15872-15883.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [33] Irie K, Zalzal S, Ozawa H, McKee MD, Nanci A: Morphological and
2
3 immunocytochemical characterization of primary osteogenic cell cultures derived
4
5 from fetal rat cranial tissue. Anat Rec 1998 ;252:554–567.
6
7
- 8 [34] Kasugai S, Shibata S, Suzuki S, Susami T, Ogura H: Characterization of a system
9
10 of mineralized-tissue formation by rat dental pulp cells in culture. Arch Oral Biol
11
12 1993;38:767–769.
13
14
- 15 [35] Foster BL, Soenjaya Y, Nociti FH Jr, Holm E, Zervas PM, Wimer HF, et al.:
16
17 Deficiency in acellular cementum and periodontal attachment in bsp null mice. J
18
19 Dent Res 2013;92:166-172.
20
21
- 22 [36] Terkeltaub R: Inorganic pyrophosphate (PPi) generation and disposition in
23
24 pathophysiology. Am J Physiol Cell Physiol 2001;281:C1–C11.
25
26
- 27 [37] Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, et al.:
28
29 Diversity, topographic differentiation, and positional memory in human
30
31 fibroblasts. Proc Natl Acad Sci U S A 2002;99:12877-12882.
32
33
- 34 [38] Passi A, Albertini R, Baccarani-Contri M, deLuca G, dePaepe A, Pallavicini G, et
35
36 al.: Proteoglycan alterations in skin fibroblast cultures from patients affected with
37
38 pseudoxanthoma elasticum. Cell Biochem Funct 1996;14:111-120.
39
40
- 41 [39] Baccarani-Contri M, Boraldi F, Taparelli F, De Paepe A, Pasquali-Ronchetti I:
42
43 Matrix proteins with high affinity for calcium ions are associated with
44
45 mineralization within the elastic fibers of pseudoxanthoma elasticum dermis. Am
46
47 J Pathol 1996;148:569-577.
48
49
- 50 [40] Quaglino D, Sartor L, Garbisa S, Boraldi F, Croce A, Passi A, et al.: Dermal
51
52 fibroblasts from pseudoxanthoma elasticum patients have raised MMP-2
53
54 degradative potential. Biochim Biophys Acta 2005;1741:42-47.
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1 **Legend to figures**
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6 **Fig. 1. Characterization of mineral deposits in dermal fibroblasts cultured in**
7 **calcifying medium.** Mineral deposits are visualized by ESEM on PXE fibroblasts after
8 20 (A) and 40 days (C, D). Panel D shows, at higher magnification, the distribution of
9 hydroxyapatite bright deposits (*) on the surface of elongated fibroblasts (arrow). EDS
10 spectra (B) demonstrate that mineral deposits are made of calcium (Ca) and phosphate
11 (P), whose levels are proportional to the size/amount of calcification.
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23 **Fig. 2. Mineral deposition in control and PXE dermal fibroblasts cultured in**
24 **calcifying medium.** The extent of mineralization has been evaluated after von Kossa
25 staining on control and PXE fibroblast cell cultures at different time points (from 10 up
26 to 40 days). The percentage of calcified areas on the whole monolayer measured in each
27 strain at same passages is shown. The increase of mineralization in control and PXE
28 fibroblasts is time-dependent, as demonstrated by linear regression analysis (insert).
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40 **Fig. 3. Western blot for actin and S100A4 protein expression.** Confluent cells were
41 cultured for 8 hours (early time) and 20 days (late time) in standard (DMEM) and in
42 calcifying (CM) media. Control (C) and PXE (P) fibroblasts at same passages are
43 compared. A representative Western blot is shown in upper panel, whereas mean values
44 from all cells strains \pm SE are shown in histograms. ** $p < 0.01$; De (early time point in
45 DMEM), CMe (early time point in CM); Dl (late time point in DMEM), CMI (late time
46 point in CM).
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1 **Fig. 4. PPI-related gene and protein expression in control fibroblasts.** RNA and
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3 protein expression of progressive ankylosis protein homolog (ANKH/ANKH),
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5 ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPPI/PC1) and
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7 of tissue-nonspecific alkaline phosphatase isozyme (ALPL/TNAP) were evaluated by
8
9 RT-PCR and Western blot in control fibroblasts at confluence in standard medium
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11 (DMEM) (set at 1) and in calcifying medium (CM) for 8 hours (early) and 20 days (late).
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13 Data from two experiments performed in triplicate with all fibroblasts cell lines are
14
15 expressed as box-and-whisker plots showing the range of mRNA and protein expression
16
17 values. The horizontal line shows the median value for each experimental condition,
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19 boxes represent the inter-quartile range and whiskers indicate the full extent of
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21 minimum/maximum values. ** p<0.01 late vs. early times in CM; ## p<0.01 CM vs.
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23 DMEM.
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33 **Fig. 5. PPI-related gene and protein expression in PXE fibroblasts.** mRNA and
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35 protein expression of progressive ankylosis protein homolog (ANKH/ANKH),
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37 ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPPI/PC1) and
38
39 of alkaline phosphatase tissue-nonspecific isozyme (ALPL/TNAP) were evaluated by
40
41 RT-PCR and Western blot in PXE fibroblasts at confluence in standard medium
42
43 (DMEM) (set at 1) and in calcifying medium (CM) for 8 hours (early) and 20 days
44
45 (late). Data from two experiments performed in triplicate with all fibroblasts cell lines
46
47 are expressed as box-and-whisker plots showing the range of mRNA and protein
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49 expression values. The horizontal line shows the median value for each experimental
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51 condition, boxes represent the inter-quartile range and whiskers indicate the full extent
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1 of minimum/maximum values. ** p<0.01 late vs. early times in CM; # p<0.05, ##
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3 p<0.01 CM vs. DMEM.
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8 **Fig. 6. PPI-related proteins in control and PXE fibroblasts.** Control and PXE
9 fibroblasts were cultured in standard medium (DMEM) (set at 1) and in calcifying
10 medium (CM) for 8 hours (early) and 20 days (late). Expression levels of progressive
11 ankylosis protein homolog (ANKH) (A) and of ectonucleotide
12 pyrophosphatase/phosphodiesterase family member 1 (PC-1) (B), evaluated by Western
13 blot, are expressed as box-and-whisker plots. The horizontal line shows the median
14 value for each experimental condition, boxes represent the inter-quartile range and
15 whiskers indicate the full extent of minimum/maximum values. TNAP activity (C) and
16 the ratio (D) between factors increasing (PC1 and ANKH) and reducing (TNAP) PPI
17 availability are expressed as mean values \pm SE. All data are from two experiments
18 performed in triplicate with all fibroblasts cell lines at same passages. * p<0.05, **
19 p<0.01, *** p<0.001 late vs. early time point in CM; # p<0.05, ## p<0.01, ### p<0.001
20 CM vs. DMEM; § p<0.05, §§ p<0.01 PXE vs. Control in the same culture condition.
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43 **Fig. 7. Osteopontin gene and protein expression in control and PXE fibroblasts.**
44 mRNA and protein expression of osteopontin (*SPPI/OPN*) were evaluated by RT-PCR
45 and Western blot in control (A, B) and in PXE (C, D) fibroblasts cultured in standard
46 medium (DMEM) (set at 1) and in calcifying medium (CM) for 8 hours (early) and 20
47 days (late). Comparison of OPN expression in control and in PXE fibroblasts at same
48 passages is shown in panel E. Data from two experiments performed in triplicate with
49 all fibroblasts cell lines are expressed as box-and-whisker plots. The horizontal line
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1 shows the median value for each experimental condition, boxes represent the inter-
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3 quartile range and whiskers indicate the full extent of minimum/maximum values. *
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6 p<0.05 late vs. early time points in CM; # p<0.05 CM vs. DMEM; § p<0.05 PXE vs.
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8 Control in the same culture condition.
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Figure 1
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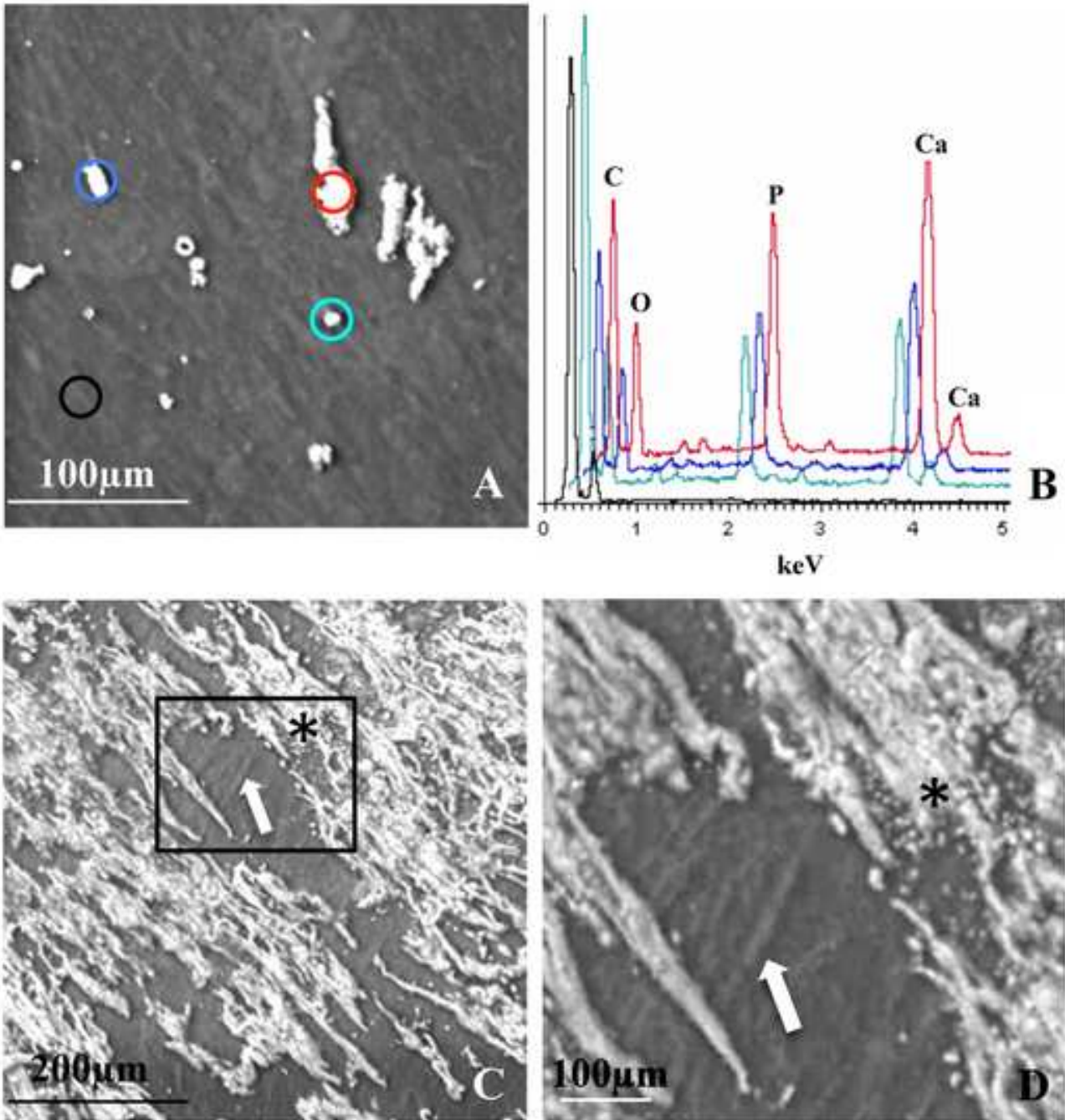


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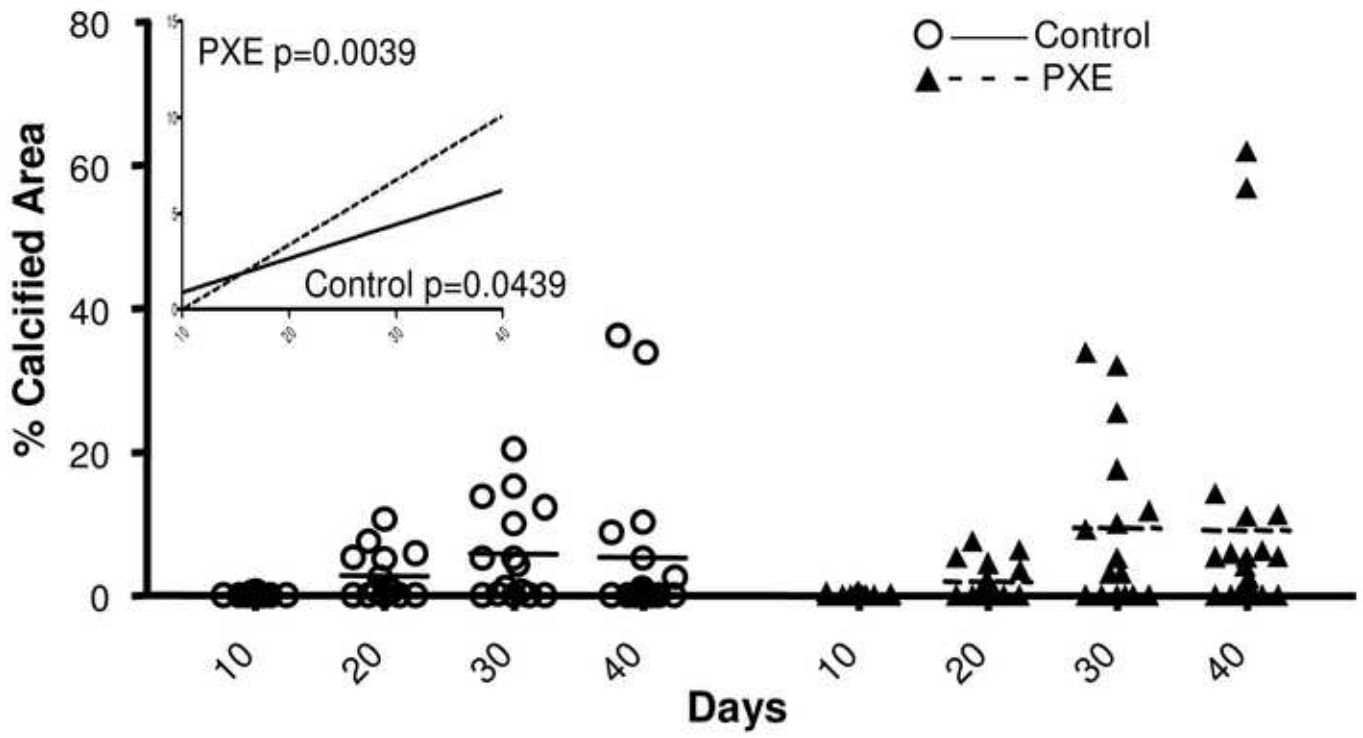


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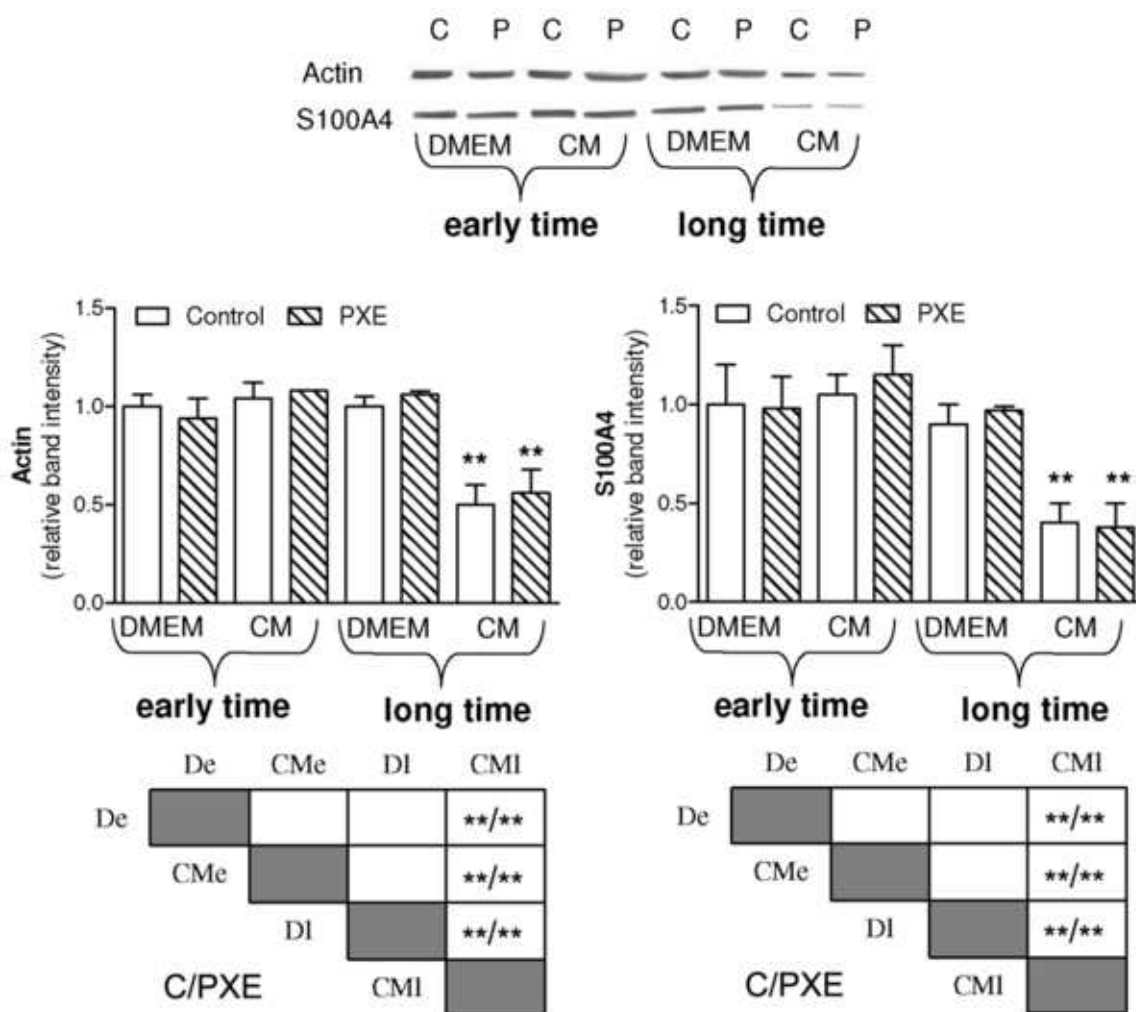


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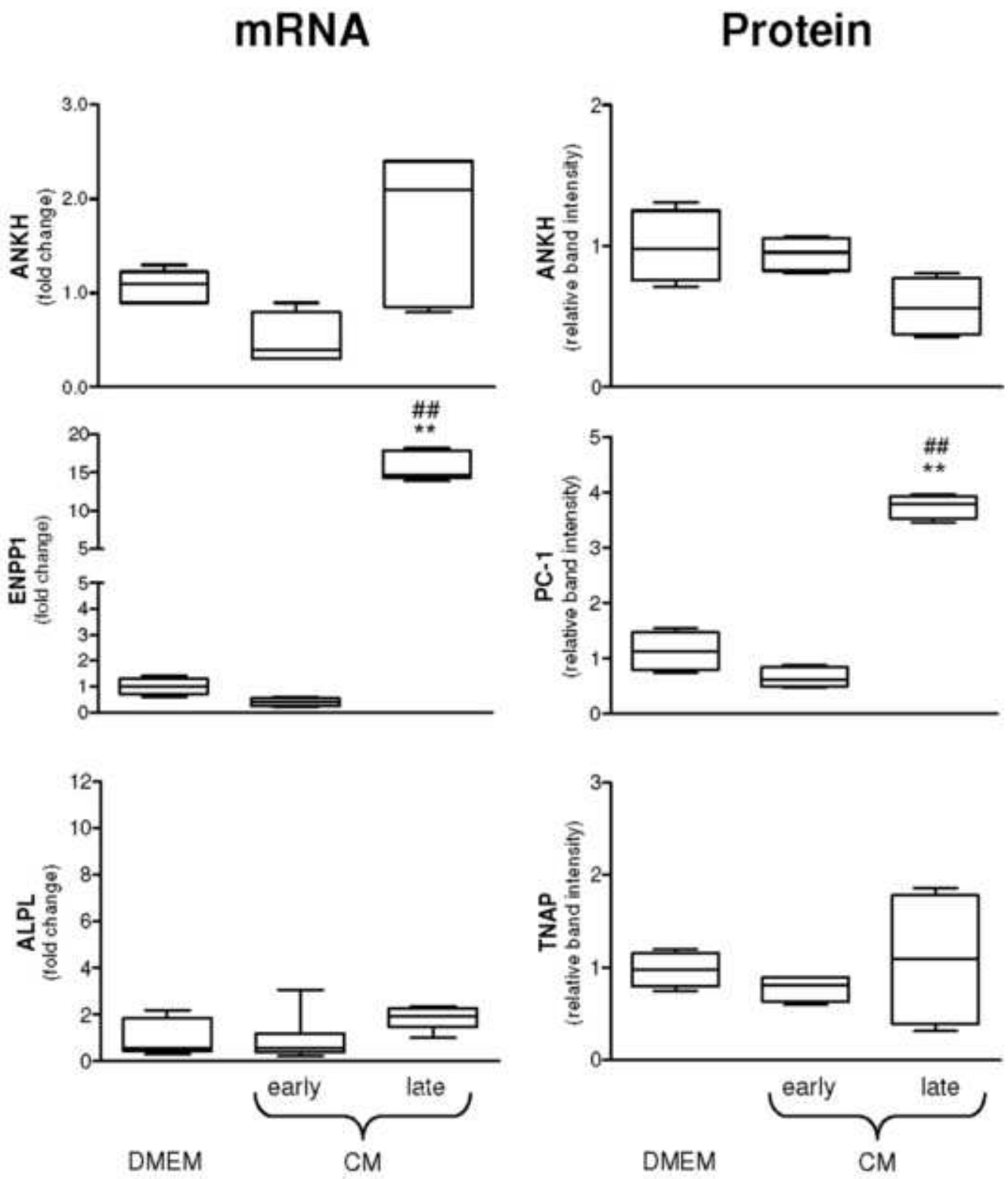


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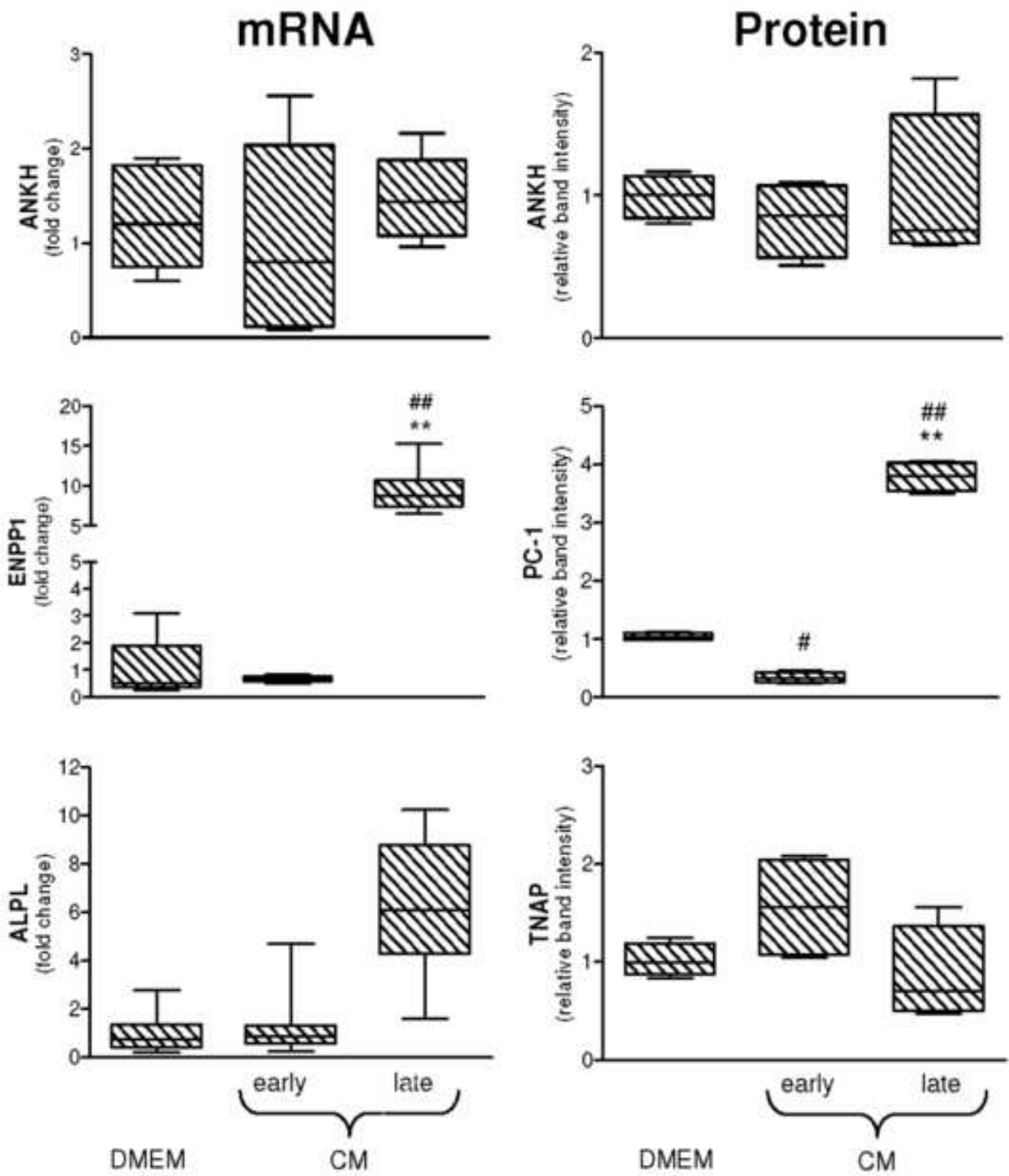


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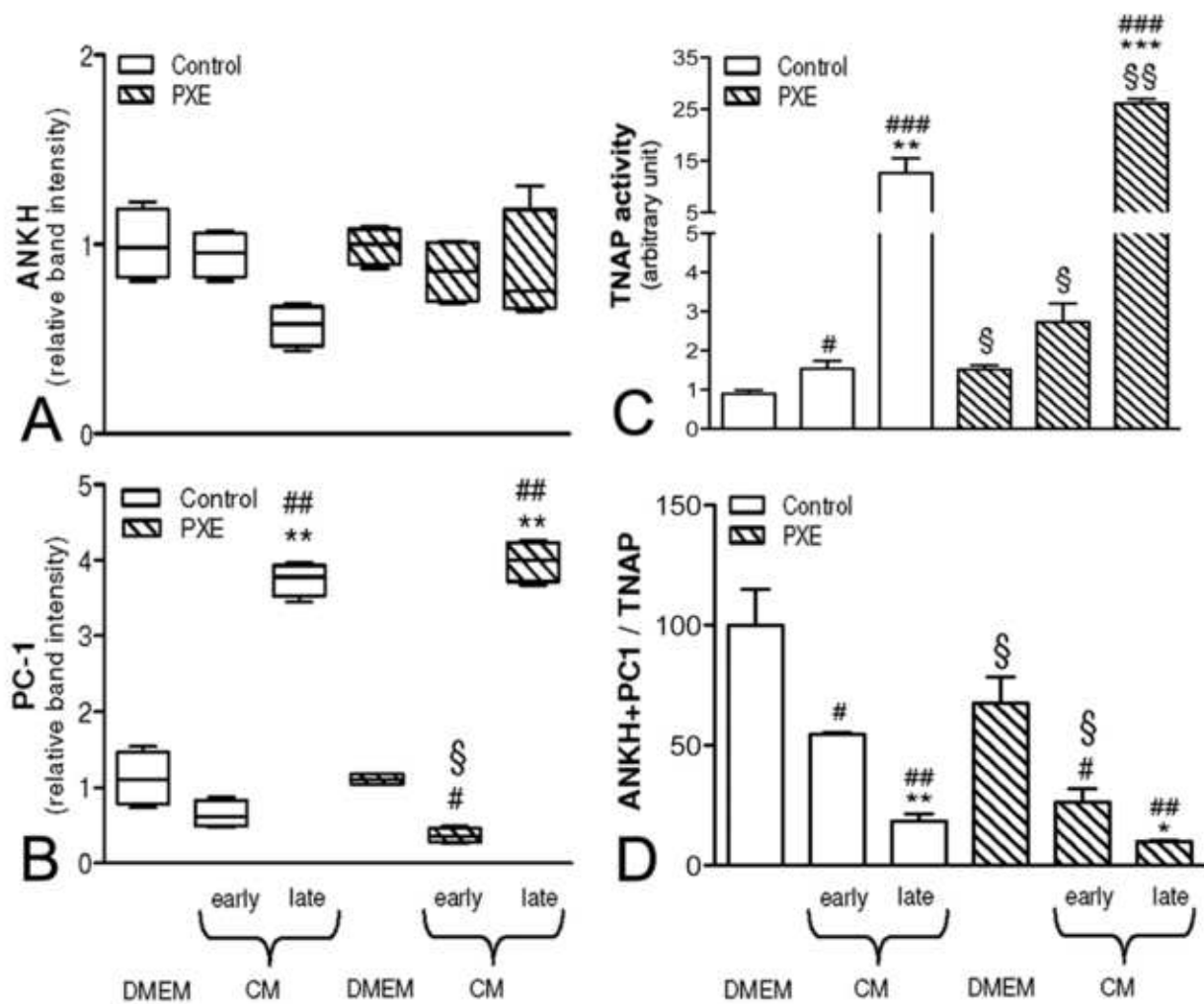


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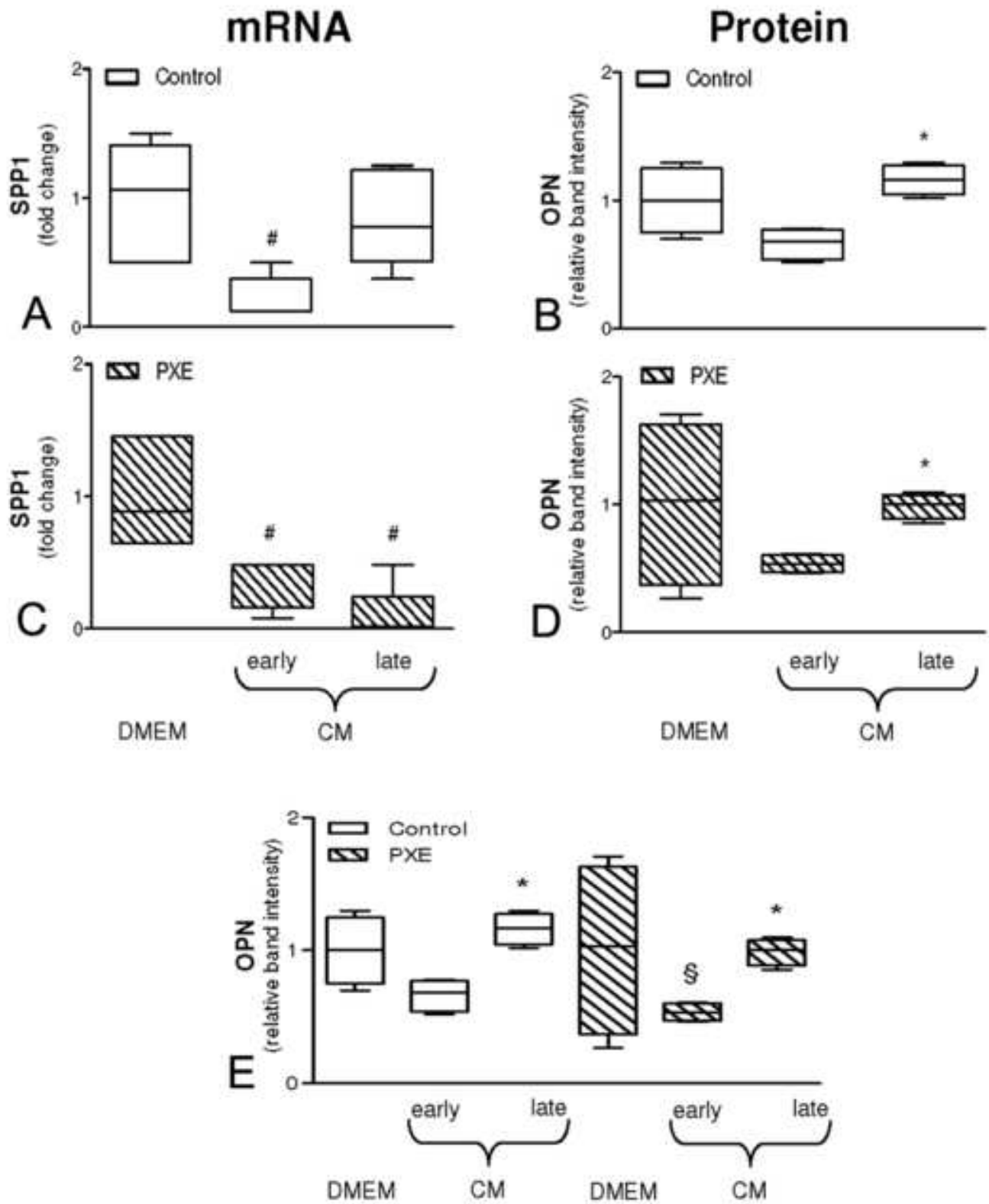


Table 1

TABLE 1. Primes used for Real Time Polymerase Chain Reaction

Gene	positions	Sequence 5'- 3'	Tm	%GC	slope	E%	R ²
ALPL forward reverse	1044-1066 1108-1131	TACAAGCACTCCCCTTCATCTG GCTCGAAGAGACCCAATAGGTAGT	60	47 50	-3.353	98.7	0.915
ANKH forward reverse	2268-2289 2327-2351	CACATGGCCGTACAAAGAGATG GTGTGGAGTAGATGGTTTCGAACTC	60	50 48	-3.426	95.8	0.920
ENPP1 forward reverse	2107-2129 2166-2192	CCGTGGACAGAAATGACAGTTTC ATGGACAGGACTAAGAGGAATTCTAAA	60	47 37	-3.207	105	0.989
SPP1 forward reverse	325-342 398-415	CTCCTAGCCCCACAGAAT GGTCATGGCTTTCGTTGG	60	56 48	-3.432	95.6	0.955
CLK2 forward reverse	1295-1313 1374-1394	GCACCATAGCACCATTGTC GCAGCCTATACTCCACACATC	60	52 52	-3.354	98.7	0.978