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Exome sequencing and bioinformatic approaches reveals rare sequence variants involved in cell signalling and elastic fibre homeostasis: new evidence in the development of ectopic calcification

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ABSTRACT

Elastic fibres undergo aberrant mineralization in genetic as well as in acquired pathologic conditions causing severe impairment of tissue mechanical properties. Despite the number of investigations performed so far, the pathogenesis of these alterations is still elusive, due to both the complexity of the elastin network and the involvement of many genes and/or pro-osteogenic signalling pathways.

Whole Exome Sequencing (WES) was performed on DNA from three patients affected by beta-thalassemia exhibiting soft connective tissue calcification. WES data were analysed with a bioinformatic approach, allowing to screen and to select genes carrying rare sequence variants. These genes were matched with those present in Extracellular Matrix DB. This approach enables to shed light on the involvement of the extracellular matrix in the occurrence of ectopic calcification.

Results revealed a number of rare sequence variants in genes related to elastic fibre assembly and integrity. For instance, the involvement of fibrillins and collagen type VI in the formation of a modified microfibrillar scaffold may lead to elastic fibres less resilient and more prone to hydroxyapatite deposition. Moreover, data reveal that changes in mitochondrial metabolic pathways are sustained by a genetic background and emphasize that a persistent chronic oxidative stress can further influence extracellular matrix homeostasis and cell signalling through the TGFβ-BMP axis. Eventually, the presence of multiple rare sequence variants in the Solute Carrier Family 25 Member 5 (SLC25A5) gene is suggestive of the role of this gene as a key factor linking mitochondria metabolism, ADP/ATP ratio and oxidative stress thus affecting extracellular matrix homeostasis and activation of pro-osteogenic factors.

1. Introduction

Ectopic calcification is an active and progressive phenomenon occurring as a frequent complication of several pathologic conditions. Interestingly, clinically relevant calcification is only rarely observed in newborns or during childhood, even in the presence of pathogenic disease-causing mutations [1]. The recent literature is disclosing an increasing number of genes and of pro-osteogenic signalling pathways contributing to the aberrant progressive deposition of hydroxyapatite [2].

Within the frame of studies investigating the mechanisms responsible for ectopic calcification, it has been observed that a number of patients affected by beta-thalassemia, independently from blood transfusion requirements, iron accumulation or chelation therapy, shows soft connective tissue mineralization responsible for the development of dermal abnormalities, cardiovascular and ophthalmological complications [3]. The genetic basis of these alterations is still unclear. *Ex-vivo* studies on skin biopsies from calcification affected areas have demonstrated changes in the extracellular components, as laterally fused collagen fibrils, presence of thread electron-dense material, fragmentation and mineralization of elastic fibres associated with accumulation of calcium-affinity glycoproteins as osteopontin, bone sialoprotein and alkaline phosphatase [4]. Additional studies on isolated and cultured fibroblasts from beta-thalassemia patients have proven that, in the presence of pathologic mineralization, cells exhibit increased anion superoxide as well as oxidized proteins and lipids maintaining, *in vitro*, the oxidative stress condition already witnessed *in vivo* in the same patients as well as in other patients with ectopic

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calcification [5,6].

Despite the number of investigations performed so far in different diseases and experimental models [1,7], it is still unclear why elastic fibres are more susceptible with respect to other matrix components to pathologic mineralization and even more elusive is the comprehension of factors regulating the variable extent of calcification in different tissues/organs or even in different areas of the same tissue.

Elastic fibres are composed of a number of molecules interacting to form a complex network required to guarantee the specific mechanical properties of connective tissues [8]. Therefore, changes in one or more of these constituents can interfere with the assembly, organization and/or stability of the whole elastic fibre. The consequences of these alterations are further worsened by the almost absent turnover of elastic fibres. Moreover, age-dependent changes in the ratio of different matrix components can favour their accumulation and/or promote metalloprotease-mediated degradation, thus contributing to the exposure of charged nucleation site for hydroxyapatite deposition [9,10].

Since it has been demonstrated that ectopic calcification is an active process with the involvement of mesenchymal cells [7,11], it can be suggested that changes in the composition of the extracellular milieu may influence connective tissue homeostasis as well as cell behaviour through modified signalling pathways [12].

Aim of the present study was to investigate in three patients affected by beta-thalassemia exhibiting soft connective tissue calcification if genes carrying rare sequence variants can be involved in molecular pathways affecting the extracellular environment and in particular elastic fibres. Whole Exome Sequencing (WES) and a bioinformatic approach were applied in order to screen for candidate genes.

2. Material and methods

2.1. Patients

In the present study, the conditions of three unrelated beta-thalassemia female patients exhibiting, at clinical examination, dermal, cardiovascular and ocular complications have been investigated.

The diagnosis of beta-thalassemia was based on the usual haematological criteria (peripheral blood evaluation and haemoglobin electrophoresis) and on the presence of mutations in *HBB* gene. Two patients (PT1 and PT2) were carriers, of a pathogenic variant in intron 1 (IVS1-110G > A) at homozygous and heterozygous status, respectively, causing a deficient beta-globin chain synthesis (β^+), while the third patient (PT3) was homozygous for a stop codon mutation in exon 2 (c.118C > T, p.Gln40*), causing absent beta-globin chain synthesis (β^0) and therefore requiring frequent blood transfusion and chelation therapy.

The study, in accordance with the basic principles of the Declaration of Helsinki, was approved by the local Ethical Committee (n. 358/17).

2.2. Light and electron microscopy

Calcification of soft connective tissues was demonstrated on dermal biopsies. Skin biopsy obtained from each patient was processed for morphological analyses as already described with modifications [13]. Briefly, samples were fixed in 10% formalin (Electron Microscopy Sciences, Hatfield, PA, USA) for light microscopy or in 2.5% glutaraldehyde (Electron Microscopy Sciences) in cacodylate buffer (Sigma-Merck, Darmstadt, D) and in 1% osmium tetroxide (Società Italiana Chimici, Roma, I) in the same buffer for electron microscopy. After dehydration, specimens were embedded in paraffin (Sigma-Merck) or in Araldite (Serva Electrophoresis, Heidelberg, D), respectively. Sections from paraffin-embedded specimens underwent von Kossa staining. Ultrathin sections (70 nm) from Araldite embedded samples were mounted on 150 mesh copper grids and contrasted with UranyLess (Electron Microscopy Sciences) followed by lead citrate staining before observation with SEM-FEG FEI Nova450 (ThermoFisher Scientific, Massachusetts, USA).

2.3. Library preparation and sequencing

DNA was extracted from whole blood using standard methods and subjected to whole exome sequencing. SureSelect Human All Exon V6′ kit (Agilent, Santa Clara, CA) was used for library preparation and exome enrichment, targeting 60 Mb of human exonic content. Samples were quantified and quality tested using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were processed with Illumina cBot (Illumina, San Diego, CA), following manufacturer's recommendations and sequenced at 9-plex level of multiplexing on NextSeq500 (Illumina, San Diego, CA), pair-end with 125 cycles per read.

2.4. Whole exome sequencing bioinformatic analysis

Raw reads were quality trimmed at both ends with erne-filter v1.4.3 [14] using default parameters and minimum read length of 40 bp. Adapters were removed by Cutadapt [15]. Trimmed reads were aligned to the human reference genome (GRCh37/hg19 assembly) with BWA [16]. Only reads that mapped to unique positions were retained and duplicated sequences were removed. GATK tools were used for realignment, base quality scores recalibration and variant discovery [17,18]. Variant functional annotation was performed by ANNOVAR [19].

We used avSNP150 database to identify known variants and corresponding rs identifiers, and RefSeq gene annotation to identify the genes harbouring each variant and its functional category. We selected only variants mapping to exons or splicing junctions of coding genes, discarding synonymous single-nucleotide variants (SNVs). Several databases (1000 Genomes Project, Exome Sequencing Project [ESP], Exome Aggregation Consortium [ExAC], Genome Aggregation Database [gnomAD]) were used to obtain variant frequency data. Only rare variants with an allele frequency ≤ 1% were retained for subsequent analysis. Moreover, we used FLAGS [20] and GDI [21] databases to discard variant in frequently mutated genes, *i.e.* variant with FLAGS score ≥ 250 or GDI score ≥ 13. Finally, for each patient we selected only variant with a coverage (DP) ≥ 10 reads and a genotype quality (GQ) ≥ 40. For variant functional prediction we used dbNSFP (v3.3a) [22] database for coding variants and dbscSNV (v1.1) [23] for splice variants. For clinical interpretation we used ClinVar database (v20170905) [24] and InterVar database (v20180118) [25].

Structural predictions of EGF-like domains of fibrillin-2 and fibrillin-3 were performed using homology-modelling web tool SWISS-MODEL (https://swissmodel.expasy.org/, swiss-model template library version 2019-02-13) [26]. Fibrillin-2 EGF-like domain 5 was modelled on 1uzj.1.A fibrillin-1 template while EGF-30 like and EGF-35 like domains of fibrillin-3 on 2w86.1.A fibrillin-1 template. Structures are graphically modified with VMD software (v. 1.9.3, http://www.ks.uiuc.edu/Research/vmd/) [27] to highlight secondary structures and variants in aminoacids sequence.

Genes ontology (GO) analysis was performed using PANTHER (v.14.0, http://www.pantherdb.org/) and organism was restricted to human [28].

The interactome of genes with rare variants involved in extracellular matrix was realized using STRING (v.10.5, https://version-10-5.string-db.org) setting no > 50 interactors [29]. The network was graphically elaborate using Cytoscape (v. 3.6.1) [30].

2.5. Immunohistochemistry

To evaluate TGF-β/SMAD signalling pathways, immunohistochemistry was performed on resin embedded skin sections of three beta-thalassemia and three healthy subjects matched for age and

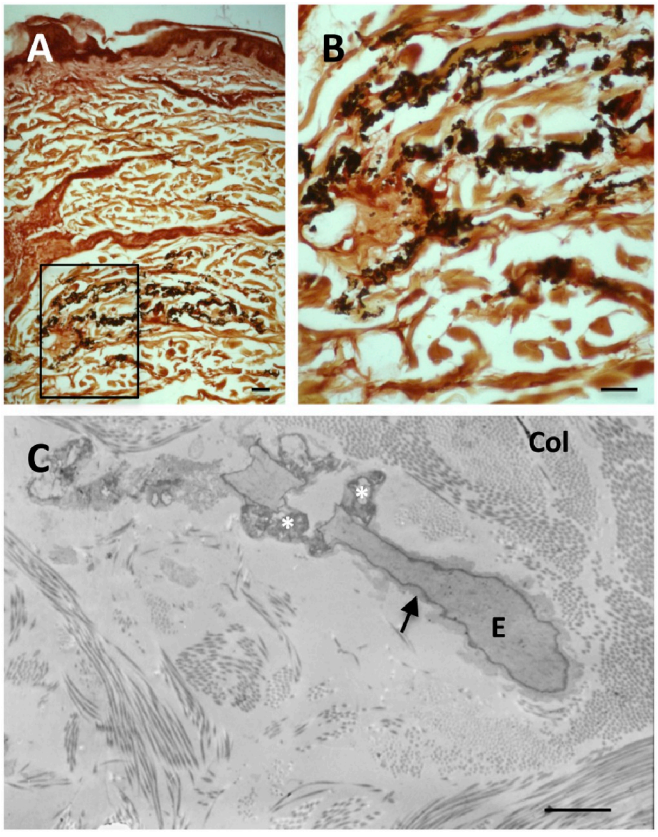

Fig. 1. Morphological analyses. (A–B) von Kossa staining of paraffin embedded skin sections shows positive (dark) mineralized areas in the reticular dermis. Bars: 10 μm. (C) Electron microscopy shows collagen bundles (Col) and elongated and calcified (arrows) as well as highly mineralized and deformed (asterisks) elastic fibres (E). Bars: 2 μm.

sex. The following primary antibodies were used: anti pSMAD1/5/8 (dilution 1:25; Merck) and anti pSMAD2/3 (dilution 1:25, Abcam) incubated at 37 °C for 1 h. As positive control liver tissue was used. Immunostaining was performed as previously described [31]. Briefly, we used the detection kit UltraView DAB on the automated system Ventana BenchMark XT (Roche, Monza, Italy). Slides were washed, mounted and evaluated using a Zeiss light microscope.

3. Results and discussion

3.1. Calcified elastic fibres

Morphological analyses of dermal biopsies from affected areas showed the presence of connective tissue alterations as previously reported [4]. In particular, von Kossa staining demonstrated the presence of mineral precipitates in specific areas of the reticular dermis (Fig. 1A, B). Transmission electron microscopy confirmed that calcification was localized on elastic fibres that appeared deformed and fragmented (Fig. 1C).

3.2. Whole exome sequencing (WES)

A bioinformatic approach was applied to WES data from three unrelated beta-thalassemia female patients with soft connective tissues calcification and exhibiting dermatological and ocular manifestations, aiming at screening for candidate gene(s) responsible for, or contributing to, elastic fibre mineralization.

In total, ~38,000 SNVs and ~1100 insertions and deletions (INDELs) variants were detected in exons and splice junctions of RefSeq coding genes. After filtering out synonymous SNVs, selecting rare variants and removing variants with low coverage and genotype quality, a total of 1963 variants (1775 SNVs and 188 INDELs) was obtained [Fig. 2A]. Almost all variants (98%) were mapped in exons and only 2% were located in splicing sites [Fig. 2B]. Most variants (83%) were classified as non-synonymous SNVs, ~5% as non-frameshift INDELs, 4% as frameshift INDELs and ~2% as stop-gain variants [Fig. 2C]. Furthermore, 272 new variants not present in the avSNP database [Fig. 2D] were identified.

In each patient ~800 variants were identified, most of which were in the heterozygous state [Fig. 3].

It is well known that complex diseases are likely caused by multiple genes and/or by multiple mutations on individual genes, however, it is becoming progressively more clear that phenotypic variability, even in apparently monogenic diseases, is the result of modifying genetic cofactors that, if mutated, are insufficient to cause a disease, but can

A

Filters	SNVs	INDELs
Select variants in exons and splice junctions of coding genes	38,300	1,176
Remove synonymous SNVs	19,807	1,176
Select rare variants	1,887	225
Remove variants with low coverage and genotype quality	1,775	188
Total variants	**1,963**	

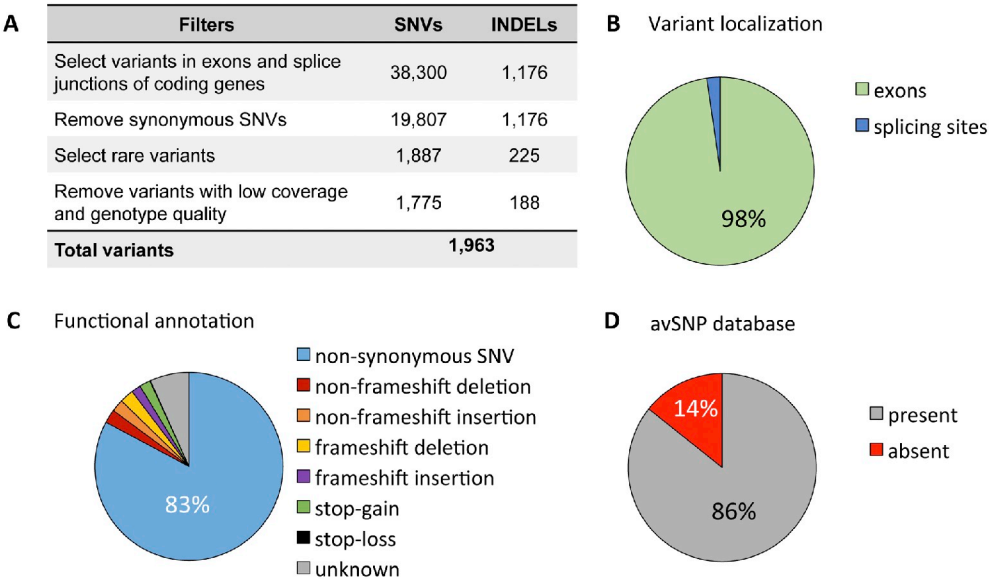

Fig. 2. Whole Exome Sequencing. A) Total number of identified rare sequence variants after subsequent filtering processes; B) rare sequence variants detected in exons or at splicing sites; C) functional annotation of rare sequence variants; D) variants present or not in the avSNP database.

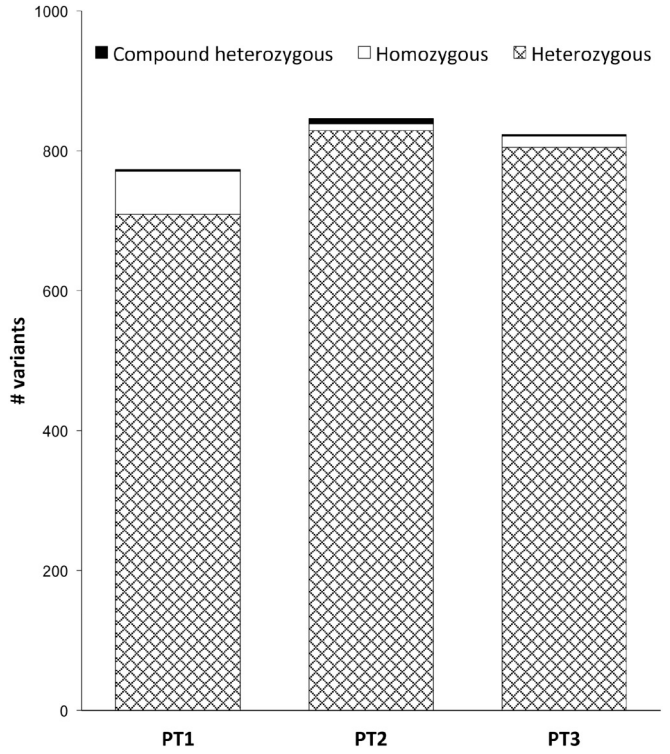

Fig. 3. Characteristics of patients' genotype. Number of rare sequence variants revealed by Whole Exome Sequencing in each patient (PT1-3).

interact with other genes to influence the susceptibility and/or the severity of a specific disease [32].

In the present study, we have focused attention only to rare sequence variants, even though we are also aware of the synergic contribution of polymorphisms, either in the same or in different genes, that may contribute to the development of clinical complications [33]. However, as a screening for candidate genes, we have preferred to apply stringent filter options focusing on rare sequence variants.

A possible limitation of this study is that we are not measuring the functional effect at protein level of a set of sequence variants, since *in silico* algorithms can only predict the effect of a single variant, although they may lead to possible false-positive as well as false-negative interpretations [34].

Nevertheless, a bioinformatic approach allows to screen for the presence of multiple sequence variants and to disclose candidate genes contributing to, or responsible for, elastic fibre calcification, thus paving the way for further studies investigating more in detail specific molecular/signalling pathways on a larger number of patients.

3.3. Extracellular-related rare sequence variants

In order to investigate if matrix-related pathways are altered in these patients, possibly contributing to the occurrence of ectopic calcification, genes carrying rare sequence variants were matched with 1363 genes present in Extracellular Matrix DB (v. 3.4, http://matrixdb.univ-lyon1.fr/). Results are shown in Table 1 and details on sequence variants and on GO are reported in Supplementary Table S1 and Table S2, respectively.

Approximately 10 to 15 extracellular matrix (ECM) genes appeared to carry rare sequence variants in all patients. Interestingly, the more consistent alterations were related to elastic fibre homeostasis.

3.3.1. Changes in elastic fibre-related genes

Even though the elastin gene does not appear to carry any rare sequence variant, changes were observed in a number of genes related to elastic fibre assembly (FBN2, FBN3, COL6A2, COL6A6, LOXL2, VCAN), signalling (TGFbR2, LTBP3) and remodelling (MMP2, ADAMTS17) (Fig. 4), most of them coding for molecules within the elastin-microfibril interface or in association with the elastic fibre-cell interface [35].

Elastogenesis is initiated by the assembly of microfibrils serving as a scaffold for elastin deposition. Microfibrils are mainly composed from three fibrillins (FBN1, FBN2 and FBN3). Whereas FBN1 is expressed throughout postnatal growth and in adults, FBN2 and FBN3 are produced in developing foetal tissues [36], thus suggesting to play a role in the early phases of elastic fibre assembly.

Table 1
List of extracellular matrix genes presenting rare sequence variants in three beta-thalassemia patients (PT). Genes common to two or three patients (PT) are highlighted in bold.

Extracellular matrix genes		
PT1	PT2	PT3
CLEC14A	ADAMTS17	COL11A2
COL7A1	AMTN	COL19A1
COLQ	CDH13	COL6A6
DST	COL15A1	CPA6
FBN3	COL16A1	CPZ
HMCN1	COL4A6	CRISPLD2
IGFALS	COL6A2	DPT
LAMA1	DST	**FBN3**
LRFN1	FBN2	FNDC8
LTBP3	FREM1	GAS6
PFKP	HSPB1	LAMA5
POSTN	LAMC3	**LTBP3**
SLC25A5	LOXL2	MMP2
SNED1	**OTOG**	**OTOG**
TSKU	PCOLCE2	**SLC25A5**
VWF	PODN	SVEP1
	PRG4	TFF3
	SLC25A5	TLR9
	SMOC2	VWA7
	TGFBR2	
	USH2A	
	VCAN	

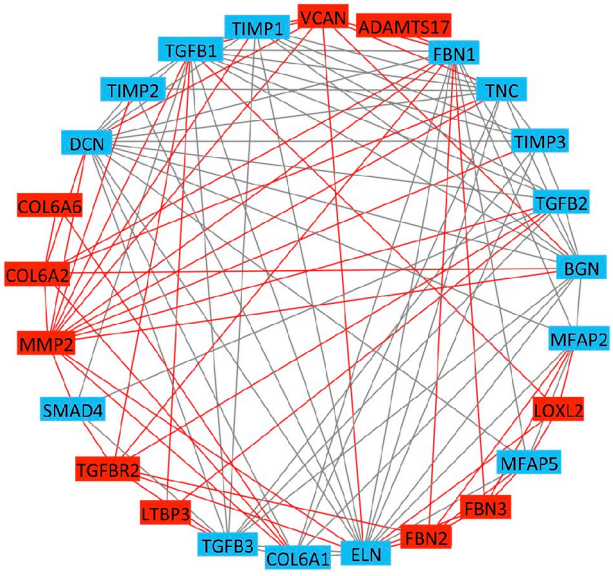

Fig. 4. Elastin interactome. Genes involved in the development/formation/degradation of elastic fibres and presenting a rare sequence variant in at least one patient are shown in red. The interactome was obtained using STRING. Network was graphically elaborated using Cytoscape. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

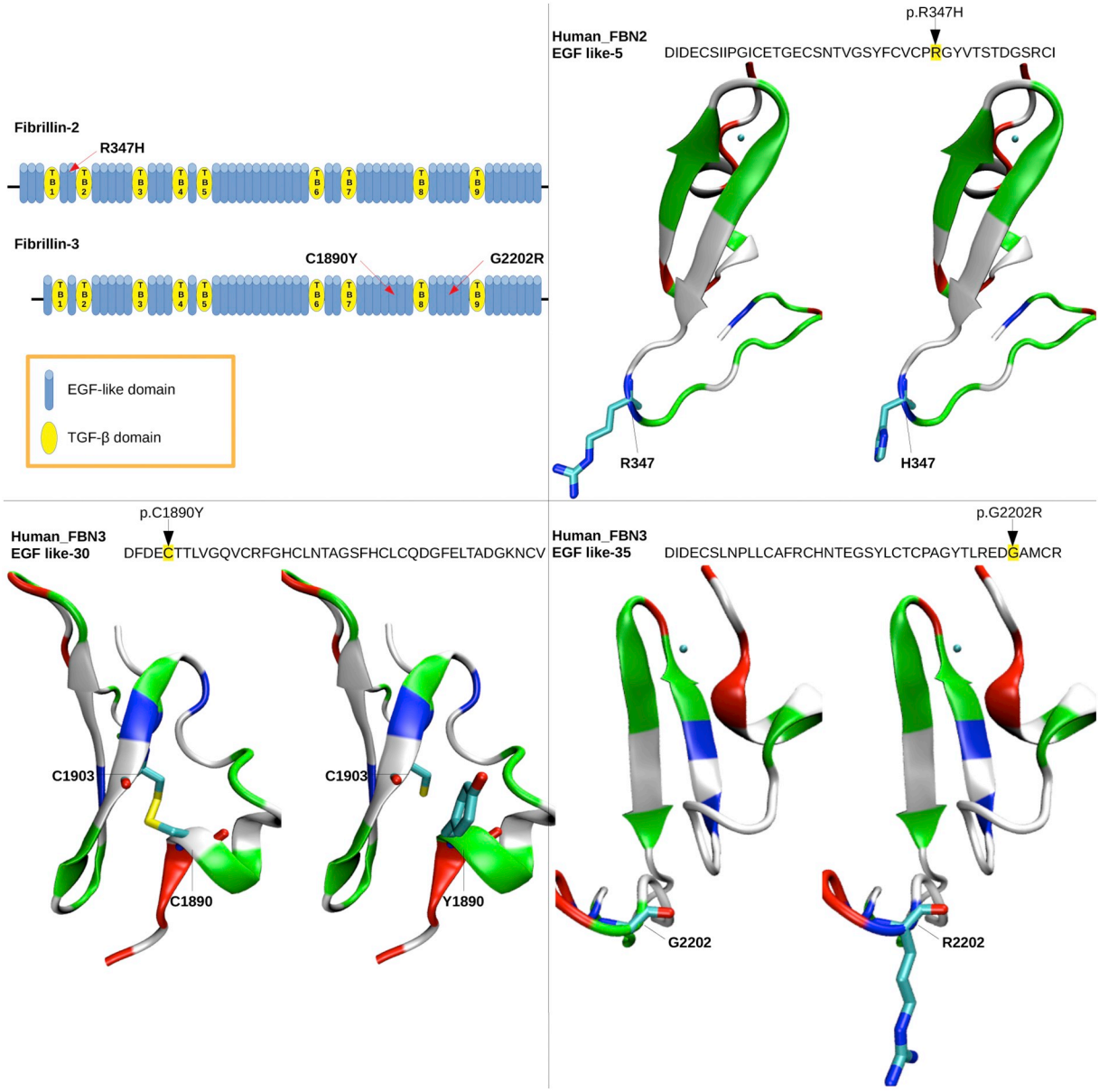

Fig. 5. Fibrillin EGF-like domain predictions. Schematic representation of fibrillin-2 and fibrillin-3 putative domains. Structural predictions of EGF-like domain 5 of human fibrillin-2, EGF-like domain 30 and EGF-like domain 35 of human fibrillin 3. Rare variants involved in amino acids changes are represented as Licorice VMD style.

In PT1 and PT3, FBN3 present p.C1890Y and p.G2202R variants, respectively, whereas PT2 has an allelic variant in FBN2 (p.R347H) (Supplementary Table S1).

Fibrillins show a primary structure characterized by a defined pattern of EGF-like domains with interspersed TGF-β binding domains (TB) (Fig. 5). TB domains with flanking EGF-like domains mediate extensive intra- and inter-molecular interactions [37–39]. Rare variants in these sequences could interfere with microfibrils physical properties and with their ability to interact with other molecules.

Primary structure of fibrillin EGF-like domains contains six highly conserved Cys forming disulfide bridges (C1-3, C2-4, C5-C6) [40–42] necessary to guarantee a conformational stability in the absence of an extensive secondary structure [36]. The variant p.C1890Y produces a C1-3 disulfide bridge break interfering with the structural integrity of the antiparallel β-sheet of EGF-30 like domain [43] (Fig. 5). Moreover, this structural change is enhanced by Cys substitution with Tyr, an amino acid with different size and physic-chemical properties. The pathogenicity of this variant was supported by *in silico* analysis.

Both FBN2 (p.R347H) and FBN3 (p.G2202R) exhibit rare sequence variants in the calcium-binding consensus sequences (Ca_EGF-like domains). For instance, the presence of Arg instead of Gly in p.G2202R induces a mass reduction and introduces a positive charge in the 35 Ca_EGF-like domain. Ca^{++} binding in a negatively charged cavity improves the fold stability and helps to secure a relative orientation of two neighbouring Ca_EGF domains. Therefore, the introduction of a positive charge in the negatively charged Ca^{++} binding cavity can likely diminish the fibrillin Ca^{++} binding affinity. Consistently, *in vitro* experiments have clearly demonstrated that calcium plays a central role in the supramolecular organization of fibrillins within microfibrils and in their functional properties, thus influencing cell-matrix interactions and cell spreading [44].

These data suggest that local changes in the environment may interfere with elastic fibre stability thus favouring accumulation of matrix components within the fibre and/or promoting degradation and fragmentation of the elastic component. It has to be noted that, even though calcified elastic fibres can be observed in many organs [45], not all

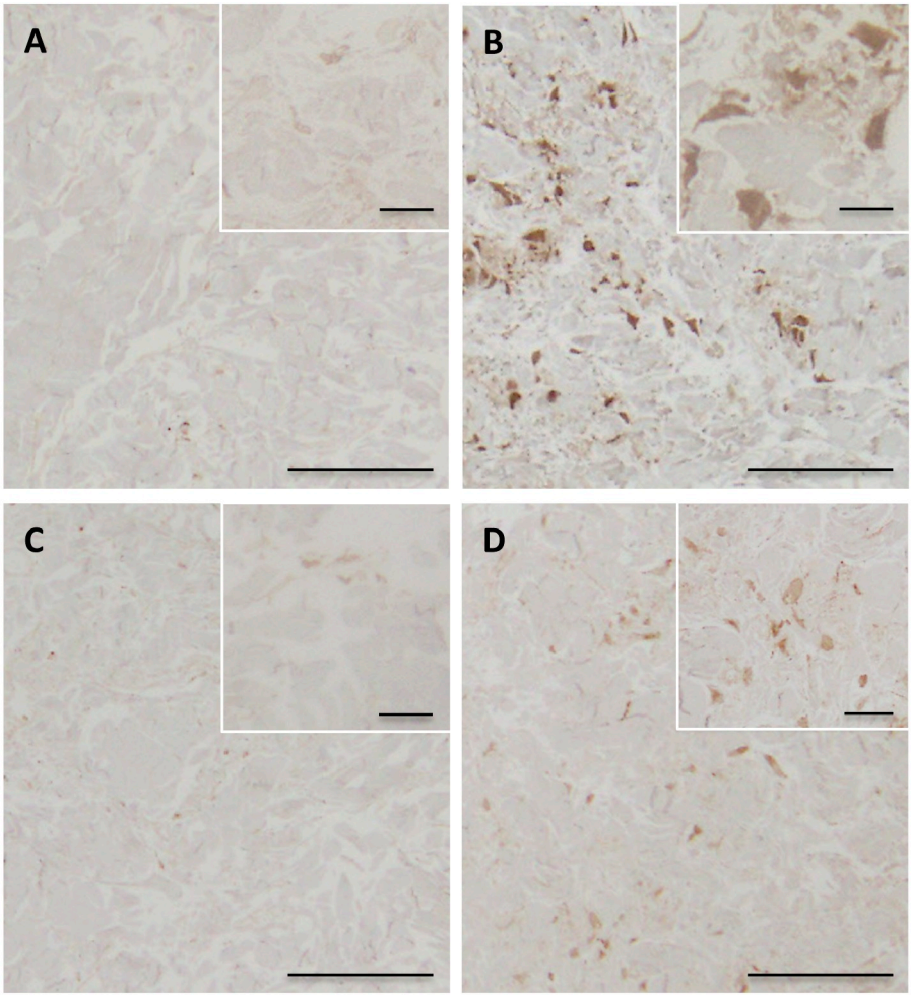

Fig. 6. Immunohistochemistry for pSMAD2/3 (A, B) and for pSMAD1/5/8 (C, D) in skin samples from control subjects (A, C) and from beta[HYPHEN]thalassemia patients with calcified elastic fibres (B, D). Scale bar = 100 μm. Insert = 50 μm.

elastic fibres are mineralized, even in patients with genetically-based ectopic calcification (*e.g. Pseudoxanthoma elasticum, PXE*) [46].

Previous investigation on elastic fibre assembly demonstrated the role of proteoglycans as carriers of tropoelastin moieties on the microfibrillar scaffold [47] and that altered networking of elastic fibre components interferes with the presence/accumulation of glycosaminoglycans (GAGs) within the fibres [48–50]. Moreover, since the type of GAGs may vary depending on several factors (*i.e.* age, organs and localization within tissues) [51], the amount and composition of GAGs within elastic fibres [47,52] may expose modified charges favouring Ca/P precipitation, thus explaining the different propensity of fibres to calcify, even within the same tissue.

In addition, changes in the composition/assembly of elastic fibres favour the increased susceptibility to metalloproteases [10] and the release of fragments that can either increase the activity of the pro-osteogenic alkaline phosphatase and either reduce the expression of the anti-osteogenic matrix GLA protein [9].

Similarly to fibrillins, type VI collagen forms a network of beaded microfilaments in the extracellular matrix. Moreover, it is negatively charged and its amount and morphology are modulated by physiological concentrations of divalent cations as Ca^{++}. However, in contrast to fibrillins, type VI collagen microfibrils are more amphiphilic and could be more responsive even to small changes in the concentration of divalent cations [44].

The collagen type VI rare sequence variants p.P414S and p.R316fs* observed in two patients can cause conformational protein changes, thus altering functionally-relevant domains.

Collagen type VI plays a structural role supporting integrin-mediated cell attachment and spreading [53], but can also promote cell signalling, hence contributing to stemness maintenance and providing cytoprotective effects, even in the presence of oxidative damage [54].

Moreover, type VI collagen microfibrils favour platelet adhesion and aggregation, and can modulate the platelet response to blood vessel injury [55]. A number of studies have clearly demonstrated that hemodynamic forces, acting on both endothelial and smooth muscle cells, regulate vascular homeostasis, translating biomechanical stimuli into biologic responses through cellular signalling pathway activation. During normal vascular homeostasis, laminar shear stress maintains an anti-inflammatory and anti-atherogenic phenotype of endothelial cells. By contrast, low shear stress and high pressure induce proinflammatory cytokine expression, cell activation and differentiation, as well as extracellular matrix reorganization. Consistently, in the case of vascular damages, platelet infiltrates/aggregates have been shown to favour the osteogenic differentiation of mesenchymal cells [56]. Endothelial cells with a pro-osteogenic phenotype have been demonstrated to be involved in the endothelial damages occurring in patients affected by soft connective tissue calcification [57]. It could be therefore suggested that, in beta-thalassemia patients, changes in the microfibrillar structure of collagen type VI may have profound consequences on cell and protein interactions, altering extracellular matrix assemblies that function as reservoir for soluble signalling molecules [58].

Fibrillin-rich microfibrils control extracellular matrix formation and

Table 2
List of mitochondria-related genes presenting rare sequence variants in three beta-thalassemia patients (PT). Genes common to two or three patients (PT) are highlighted in bold.

Mitochondria-related genes		
PT1	PT2	PT3
ACAD10	AGK	ABCA13
ACADL	ALDH1B1	ADCK1
CBR4	ALKBH3	ARG2
CS	ANGEL2	CKMT1B
FDPS	ATIC	CYB5R2
MRPL13	ATP10D	FAM136A
NEU4	CPT2	GOLPH3
NIT2	ETFB	**GPX1**
PDE12	GCAT	HEMK1
SLC25A5	**GPX1**	IBA57
TOP3A	IDE	IMMT
	KIF1B	MECR
	KRT5	MRPL46
	MCAT	MTG1
	MMACHC	NME3
	MPST	OBSCN
	MTCH2	SLC25A47
	PITRM1	**SLC25A5**
	PRODH	TRIAP1
	PRSS35	
	SLC25A27	
	SLC25A5	
	TIMM44	
	WARS2	

remodelling by regulating, in a context-specific temporal (developmental) and spatial (tissue-specific) manner, the storage, diffusion and release of several molecules such as BMPs, TGFβ, fibulins and the versican–hyaluronan proteoglycan complex [59–63]. TGFβ is secreted from cells as a latent complex consisting of TGFβ, the TGFβ pro-peptide, and a molecule of latent TGFβ binding protein (LTBP). LTBPs play a crucial role in the folding and secretion of TGFβ, and in the localization of these complexes into specific sites of the ECM [64–66]. Moreover, LTBP-3 controls TGFβ levels during the commitment phase of mesenchymal stem cell differentiation to osteoblasts [67,68].

In two patients, LTBP3 presents p.G36delinsLLLG variant that falls within the hydrophobic region (H-region) of the signal peptide. Several studies have demonstrated that signal peptide variations alter translocation efficiency, cleavage sites and post-cleavage events. In particular, p.G36delinsLLLG increases the hydrophobicity of the H-region by introducing three Leu. Interfering with H-region hydrophobicity can affect the secretion of the mature protein, the rate and efficiency of protein translocation as well as protein expression by changing the quantity of the mRNA at steady state by forming different mRNA secondary structures [69,70] and consequently interfere with TGFβ availability.

Alterations in this control system have been linked to the pathogenesis of various diseases, associated to modifications in tissue morphology and function [71]. Consistently, previous observation in *Pseudoxanthoma elasticum (PXE)*, a rare genodermatosis characterized by ectopic calcification [72], demonstrated that changes in TGFβ-related pathways can affect bone morphogenetic protein (BMP) signalling [73].

BMPs, members of the TGFβ superfamily, were initially deemed to specifically induce new bone formation [74]. However, additional studies demonstrated that BMPs play important roles also in soft connective tissues. BMP-2 and BMP-4, for instance, regulate many cellular functions including cardiovascular development, angiogenesis, and smooth muscle cell chemotaxis in response to vascular injury and calcification [75,76]. BMP2 undergoes a complex regulatory mechanism by Matrix Gla Protein (MGP) [77]. Low levels of MGP relative to BMP2 may result in mild enhancement of BMP2 activity, whereas intermediate levels would inhibit, and high levels strongly increase, BMP2 activity [7]. These findings clearly demonstrate the complexity of the mechanisms regulating ectopic calcification, which depend not only on the presence/absence of specific proteins and on their activity (due, for instance, to post-translational modifications, as phosphorylation and carboxylation), but also on the ratio among different molecules.

Since carboxylated MGP is reduced in beta-thalassemia [5], it can be suggested that altered signalling pathways can interfere with the TGFβ-BMP axis, thus promoting and sustaining pathologic calcification.

In order to verify if SMAD signalling pathways are modified in the presence of ectopic calcification we have performed immunohistochemistry assays on skin sections. In the calcified skin of beta-thalassemia patients, fibroblasts were abundantly present in the dermis, exhibited a large and spread morphology and were strongly positive for pSMAD2/3, clearly indicating an activation of this signalling pathway (Fig. 6B). On the contrary, in healthy skin, fibroblasts were less numerous and with a weak positivity (Fig. 6A). Consistently, recent findings have shown that pSMAD2/3 accumulates in vessel wall calcification [78].

Similar results were also observed for the pSMAD1/5/8 signalling pathway (Fig. 6C and D). These findings are in agreement with those obtained in PXE patients, typically characterized by elastic fibre calcification [72], where pSMAD1/5/8 up-regulates the pro-osteogenic runt-related transcriptional factor2 (Runx2), thus inducing the expression of pro-calcifying genes (*i.e.* osterix, alkaline phosphatase).

These data indicate that aberrant mineral deposition is related to changes in signalling pathways involving TGFβ-BMP axis.

3.4. Altered extracellular matrix may affect energy-metabolic properties

Further looking at genes present in the Extracellular Matrix DB that were carrying rare sequence variants in our patients, SLC25A5, also known as ANT2, appeared to be the most frequently affected.

ANTs are mitochondrial carrier proteins that exchange ATP and ADP between the cytoplasm and the mitochondrial matrix, but ANTs are also located at the plasma membrane of fibroblasts [79–81] and, by interacting with the membrane-bounded protease MT1-MMP, link energy metabolism to pericellular proteolysis [79].

ANT2, in particular, regulates the ADP/ATP ratio in mitochondrial oxidative phosphorylation.

The relative concentration of ADP, ATP, and consequently inorganic pyrophosphate, are linked to P2 purinergic receptor activation, suggesting that they play a role in the pathogenesis of ectopic calcification in PXE [82]. In agreement with these data is the finding that P2 receptors enhance osteoblast differentiation involving PI3K/AKT signalling pathway activation and gene expression induction of alkaline phosphatase activity, bone sialoprotein and expression of osteogenic proteins BMP-2 and BMP-4 [83].

ATP production and energy metabolism are highly variable in different tissues and cells. This variability results from both the differential expression of genes involved in oxidative phosphorylation and in the kinetic of ATP and ADP exchange in the cell, further supporting the hypothesis that local factors contribute to the pro-osteogenic environment.

Furthermore, ANT2 plays important roles not only in ATP production, but also in mitochondrial membrane potential stabilization and in cellular resistance to oxidative stress [84].

The observation that all three beta-thalassemia patients are carriers of several sequence variants in the SLC25A5 gene (Supplementary Tables S3 and S4) is consistent with the altered redox balance that was previously demonstrated in patients with ectopic calcification [5,6].

Consistently, rare sequence variants were also observed in glutathione peroxidase-1 (GPx1) gene that encodes for an enzyme acting as a primary defence against free radical-mediated damages in mitochondria. Moreover, GPx1 has been associated to the occurrence of

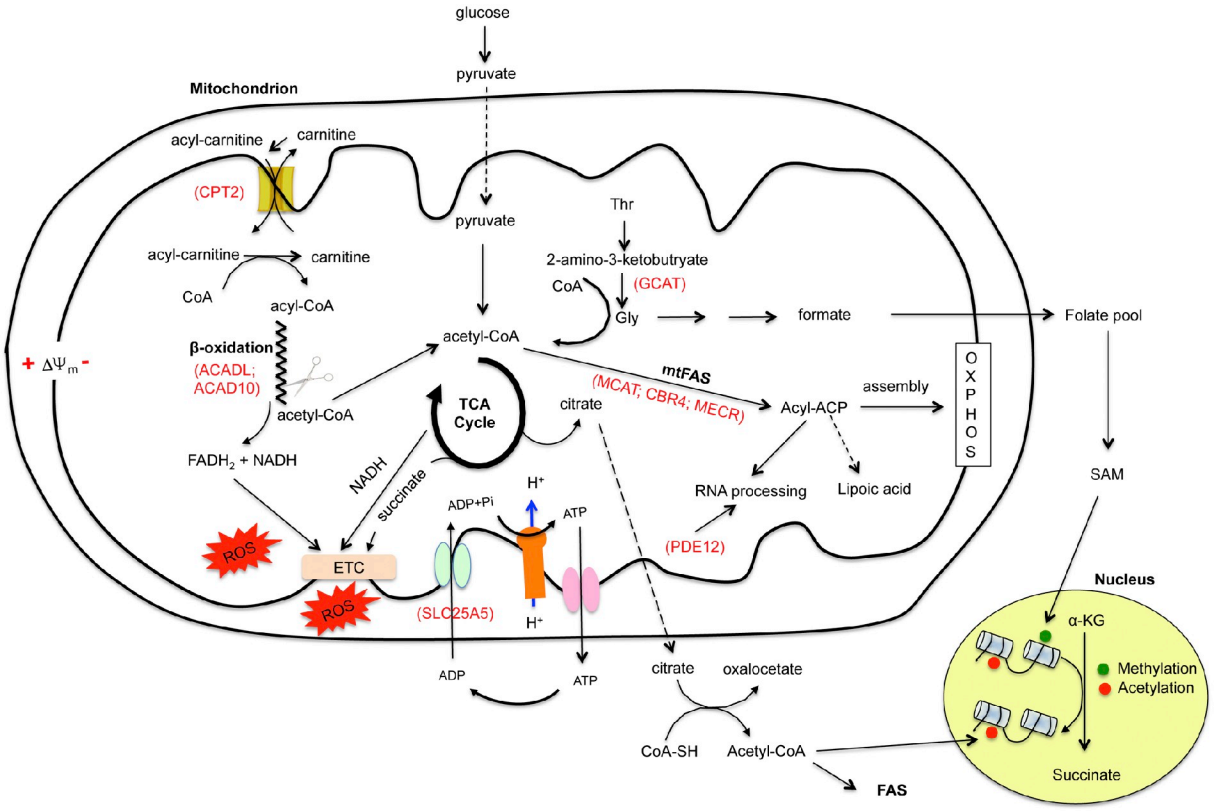

Fig. 7. Mitochondrial function and metabolism. Drawing schematically represents different interconnected pathways within mitochondria: *i.e.* oxidative phosphorylation (OXPHOS), the Krebs cycle (TCA) and the fatty acids beta-oxidation. Metabolites, electron transport chain, ATP production and its efflux from mitochondria are also shown. Genes carrying rare sequence variants are highlighted in red. ACADL = acyl-CoA dehydrogenase long chain; ACAD10 = acyl-CoA dehydrogenase family member 10; CBR4 = carbonyl reductase 4; CPT2 = carnitine palmitoyltransferase 2; ETC = electron transport chain; GCAT = glycine *C*-acetyltransferase; MCAT = malonyl-CoA-acyl carrier protein transacylase; MECR = mitochondrial trans-2-enoyl-CoA reductase; PDE12 = phosphodiesterase 12; SLC25A5 = solute carrier family 25 member 5; ROS = reactive oxygen species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ectopic calcification in genetic (i.e PXE) [85] and acquired diseases (*i.e.* type II diabetes) [86]. These data further sustain the relationship between oxidative stress and calcification.

Chronic oxidative stress condition, inflammation and subsequent secretion of metalloproteases all contribute to elastin fragility and fragmentation, especially in fibres characterized by loss of flexibility and altered conformation due to the presence of carbamylation products and aldehydes generated from oxidative stress-induced peroxidation of poly-unsaturated fatty acids [87].

In addition to SLC25A5, numerous genes involved in mitochondrial energy metabolism appeared to carry rare sequence variants in patients (Table 2 and Supplementary Tables S3 and S4).

Fig. 7 shows a number of genes involved in long-chain fatty acid β-oxidation (FAO) or in mitochondria fatty acid synthesis (mtFAS). It has been previously reported that enzymes involved in FAO and electron transport chain (ETC) are physically associated [88] and therefore altered FAO enzymatic activities can induce accumulation of long-chain fatty acids causing mitochondrial dysfunction by different ways such as calcium homeostasis disturbance, mitochondrial membrane potential dissipation, OXPHOS uncoupling and apoptosis [89].

4. Conclusion

A bioinformatic approach was applied to WES data from three patients affected by beta-thalassemia and ectopic calcification, aiming at disclosing candidate gene(s) that in the extracellular environment can modulate cell behaviour and signalling pathways responsible for, or contributing to, elastic fibre mineralization.

In the light of these results, it can be suggested that a number of rare sequence variants can modify elastic fibre packaging, permeability and/or flexibility, thus weakening its stability. Moreover, less compact elastic fibres can be either more susceptible to proteolysis and fragmentation or may accumulate charged matrix components as GAGs, creating a more suitable pro-osteogenic environment.

Changes in elastic fibre assembly and stability can interfere with TGF-beta availability as indicated by activated TGFβ-BMP signalling pathways favouring ectopic calcification.

Eventually, altered extracellular matrix homeostasis is also linked to oxidative stress and to changes in mitochondrial energy metabolism leading to modified ADP/ATP ratio, suggesting that these changes can modulate osteogenic differentiation of mesenchymal cells.

Conflicts of interest

None.

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Author contributions

F.B. and D.Q. designed experiments and wrote the manuscript; F.B., F.D.L. and P.M. performed experiments and data analysis; O.R. and A.G. set up and performed bioinformatics analyses; L.L. performed immunohistochemistry assay; S.B. critically supervised bioinformatic analyses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.020.

References

[1] Q. Li, Q. Jiang, J. Uitto, Ectopic mineralization disorders of the extracellular matrix of connective tissue: molecular genetics and pathomechanisms of aberrant calcification, Matrix Biol. 33 (2014) 23–28, https://doi.org/10.1016/j.matbio.2013.06.003.

[2] M. Bäck, T. Aranyi, M.L. Cancela, M. Carracedo, N. Conceição, G. Leftheriotis, V. Macrae, L. Martin, Y. Nitschke, A. Pasch, D. Quaglino, F. Rutsch, C. Shanahan, V. Sorribas, F. Szeri, P. Valdevielso, O. Vanakker, H. Kempf, Endogenous calcification inhibitors in the prevention of vascular calcification: a consensus statement from the COST action EuroSoftCalcNet, Front. Cardiovasc. Med. 5 (2019) 196, https://doi.org/10.3389/fcvm.2018.00196.

[3] A. Aessopos, D. Farmakis, D. Loukopoulos, Elastic tissue abnormalities resembling pseudoxanthoma elasticum in beta thalassemia and the sickling syndromes, Blood 99 (2002) 30–35, https://doi.org/10.1182/blood.V99.1.30.

[4] M. Baccarani-Contri, B. Bacchelli, F. Boraldi, D. Quaglino, F. Taparelli, E. Carnevali, M.A. Francomano, S. Seidenari, V. Bettoli, V. De Sanctis, I. Pasquali-Ronchetti, Characterization of pseudoxanthoma elasticum-like lesions in the skin of patients with beta-thalassemia, J. Am. Acad. Dermatol. 44 (2001) 33–39, https://doi.org/10.1067/mjd.2001.110045.

[5] F. Boraldi, M. Garcia-Fernandez, C. Paolinelli-Devincenzi, G. Annovi, L. Schurgers, C. Vermeer, P. Cianciulli, I. Pasquali-Ronchetti, D. Quaglino, Ectopic calcification in β-thalassemia patients is associated with increased oxidative stress and lower MGP carboxylation, Biochim. Biophys. Acta 1832 (2013) 2077–2084, https://doi.org/10.1016/j.bbadis.2013.07.017.

[6] I. Pasquali-Ronchetti, M.I. Garcia-Fernandez, F. Boraldi, D. Quaglino, D. Gheduzzi, Paolinelli C. De Vincenzi, R. Tiozzo, S. Bergamini, D. Ceccarelli, U. Muscatello, Oxidative stress in fibroblasts from patients with pseudoxanthoma elasticum: possible role in the pathogenesis of clinical manifestations, J. Pathol. 208 (2006) 54–61, https://doi.org/10.1002/path.1867.

[7] I. Ronchetti, F. Boraldi, G. Annovi, P. Cianciulli, D. Quaglino, Fibroblast involvement in soft connective tissue calcification, Front. Genet. 4 (2013) 22, https://doi.org/10.3389/fgene.2013.00022.

[8] A.K. Baldwin, A. Simpson, R. Steer, S.A. Cain, C.M. Kielty, Elastic fibres in health and disease, Expert Rev. Mol. Med. 15 (2013) e8, , https://doi.org/10.1017/erm.2013.9.

[9] S. Wanga, S. Hibender, Y. Ridwan, C. van Roomen, M. Vos, I. van der Made, N. van Vliet, R. Franken, L.A. van Riel, M. Groenink, A.H. Zwinderman, B.J. Mulder, C.J. de Vries, J. Essers, V. de Waard, Aortic microcalcification is associated with elastin fragmentation in Marfan syndrome, J. Pathol. 243 (2017) 294–306, https://doi.org/10.1002/path.4949.

[10] D.M. Basalyga, D.T. Simionescu, W. Xiong, B.T. Baxter, B.C. Starcher, N.R. Vyavahare, Elastin degradation and calcification in an abdominal aorta injury model: role of matrix metalloproteinases, Circulation 110 (2004) 3480–3487, https://doi.org/10.1161/01.CIR.0000148367.08413.E9.

[11] T. Kirsch, Biomineralization-an active or passive process? Connect. Tissue Res. 53 (2012) 438–445, https://doi.org/10.3109/03008207.2012.730081.

[12] L. Hortells, S. Sur, C. St Hilaire, Cell phenotype transitions in cardiovascular calcification, Front. Cardiovasc. Med. 5 (2018) 27, https://doi.org/10.3389/fcvm.2018.00027.

[13] D. Taverna, F. Boraldi, G. De Santis, R.M. Caprioli, D. Quaglino, Histology-directed and imaging mass spectrometry: an emerging technology in ectopic calcification, Bone 74 (2015) 83–94, https://doi.org/10.1016/j.bone.2015.01.004.

[14] C. Del Fabbro, S. Scalabrin, M. Morgante, F.M. Giorgi, An extensive evaluation of read trimming effects on Illumina NGS data analysis, PLoS One 8 (2013) e85024, , https://doi.org/10.1371/journal.pone.0085024.

[15] M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads, EMBnet J. 17 (2011) 10–12, https://doi.org/10.14806/ej.17.1.200.

[16] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler Transform, Bioinformatics 25 (2009) 1754–1760, https://doi.org/10.1093/bioinformatics/btp324.

[17] M.A. DePristo, E. Banks, R. Poplin, K.V. Garimella, J.R. Maguire, C. Hartl, A.A. Philippakis, G. del Angel, M.A. Rivas, M. Hanna, A. McKenna, T.J. Fennell, A.M. Kernytsky, A.Y. Sivachenko, K. Cibulskis, S.B. Gabriel, D. Altshuler, M.J. Daly, A framework for variation discovery and genotyping using next-generation DNA sequencing data, Nat. Genet. 43 (2011) 491–498, https://doi.org/10.1038/ng.806.

[18] G.A. Van der Auwera, M.O. Carneiro, C. Hartl, R. Poplin, G. Del Angel, A. Levy-Moonshine, T. Jordan, K. Shakir, D. Roazen, J. Thibault, E. Banks, K.V. Garimella, D. Altshuler, S. Gabriel, M.A. DePristo, From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline, Curr. Protoc. Bioinformatics 43 (2013) 11.10.1–33, https://doi.org/10.1002/0471250953.bi1110s43.

[19] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants from next-generation sequencing data, Nucleic Acids Res. 38 (2010) e164, , https://doi.org/10.1093/nar/gkq603.

[20] C. Shyr, M. Tarailo-Graovac, M. Gottlieb, J.J.Y. Lee, C. Van Karnebeek, W.W. Wasserman, FLAGS, frequently mutated genes in public exomes, BMC Med. Genet. 7 (2017) 64, https://doi.org/10.1186/s12920-014-0064-y.

[21] Y. Itan, L. Shang, B. Boisson, E. Patin, A. Bolze, M. Moncada-Vélez, E. Scott, M.J. Ciancanelli, F.G. Lafaille, J.G. Markle, R. Martinez-Barricarte, S.J. de Jong, X.F. Kong, P. Nitschke, A. Belkadi, J. Bustamante, A. Puel, S. Boisson-Dupuis, P.D. Stenson, J.G. Gleeson, D.N. Cooper, L. Quintana-Murci, J.M. Claverie, S.Y. Zhang, L. Abel, J.L. Casanova, The human gene damage index as a gene-level approach to prioritizing exome variants, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 13615–13620, https://doi.org/10.1073/pnas.1518646112.

[22] X. Liu, C. Wu, C. Li, E. Boerwinkle, dbNSFP v3.0: a one-stop database of functional predictions and annotations for human non-synonymous and splice site SNVs, Hum. Mutat. 37 (2016) 235–241, https://doi.org/10.1002/humu.22932.

[23] X. Jian, E. Boerwinkle, X. Liu, In silico prediction of splice-altering single nucleotide variants in the human genome, Nucleic Acids Res. 42 (2014) 13534–13544, https://doi.org/10.1093/nar/gku1206.

[24] M.J. Landrum, J.M. Lee, M. Benson, G. Brown, C. Chao, S. Chitipiralla, B. Gu, J. Hart, D. Hoffman, J. Hoover, W. Jang, K. Katz, M. Ovetsky, G. Riley, A. Sethi, R. Tully, R. Villamarin-Salomon, W. Rubinstein, D.R. Maglott, ClinVar: public archive of interpretations of clinically relevant variants, Nucleic Acids Res. 44 (2016) D862–D868, https://doi.org/10.1093/nar/gkv1222.

[25] Q. Li, K. Wang, InterVar: clinical interpretation of genetic variants by ACMG-AMP 2015 guideline, Am. J. Hum. Genet. 100 (2017) 267–280, https://doi.org/10.1016/j.ajhg.2017.01.004.

[26] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F.T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res. 46 (2018) 296–303, https://doi.org/10.1093/nar/gky427.

[27] W. Humphrey, A. Dalke, K. Schulten, VMD - visual molecular dynamics, J. Mol. Graph. 14 (1996) 33–38.

[28] H. Mi, X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, P.D. Thomas, PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements, Nucleic Acids Res. 45 (2017) 183–189, https://doi.org/10.1093/nar/gkw1138.

[29] B. Snel, G. Lehmann, P. Bork, M.A. Huynen, STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene, Nucleic Acids Res. 18 (2000) 3442–3444.

[30] P. Shannon, A. Markie, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504, https://doi.org/10.1101/gr.1239303.

[31] F. Boraldi, L. Losi, D. Quaglino, Pigment epithelial-derived factor: a new player in the calcification of dermal elastic fibre? Br. J. Dermatol. 177 (2017) e44–e46, https://doi.org/10.1111/bjd.15223.

[32] S. Chakravorty, M. Hegde, Inferring the effect of genomic variation in the new era of genomics, Hum. Mutat. 39 (2018) 756–773, https://doi.org/10.1002/humu.23427.

[33] F. Boraldi, S. Costa, C. Rabacchi, M. Ciani, O. Vanakker, D. Quaglino, Can APOE and MTHFR polymorphisms have an influence on the severity of cardiovascular manifestations in Italian Pseudoxanthoma elasticum affected patients? Mol. Genet. Metab. Rep. 1 (2014) 477–482, https://doi.org/10.1016/j.ymgmr.2014.11.002.

[34] I.A. Adzhubei, S. Schmidt, L. Peshkin, V.E. Ramensky, A. Gerasimova, P. Bork, A.S. Kondrashov, S.R. Sunyaev, A method and server for predicting damaging missense mutations, Nat. Methods 7 (2010) 248–249, https://doi.org/10.1038/nmeth0410-248.

[35] C.M. Kielty, T.J. Wess, L. Haston, J.L. Ashworth, M.J. Sherratt, C.A. Shuttleworth, Fibrillin-rich microfibrils: elastic biopolymers of the extracellular matrix, J. Muscle Res. Cell Motil. 23 (2002) 581–596, https://doi.org/10.1023/A:102347901.

[36] M.R. Davis, K.M. Summers, Structure and function of the mammalian fibrillin gene family: implications for human connective tissue diseases, Mol. Genet. Metab. 107 (2012) 635–647, https://doi.org/10.1016/j.ymgme.2012.07.023.

[37] S.S. Lee, V. Knott, J. Jovanović, K. Harlos, J.M. Grimes, L. Choulier, H.J. Mardon, D.I. Stuart, P.A. Handford, Structure of the integrin binding fragment from fibrillin-1 gives new insights into microfibril organization, Structure 12 (2004) 717–729, https://doi.org/10.1016/j.str.2004.02.023.

[38] R.S. Smallridge, P. Whiteman, J.M. Werner, I.D. Campbell, P.A. Handford, A.K. Downing, Solution structure and dynamics of a calcium binding epidermal growth factor-like domain pair from the neonatal region of human fibrillin-1, J. Biol. Chem. 278 (2003) 12199–12206, https://doi.org/10.1074/jbc.M208266200.

[39] S.A. Jensen, I.B. Robertson, P.A. Handford, Dissecting the fibrillin microfibril: structural insights into organization and function, Structure 20 (2012) 215–225, https://doi.org/10.1016/j.str.2011.12.008.

[40] C.M. Kielty, C.A. Shuttleworth, The role of calcium in the organization of fibrillin microfibrils, FEBS Lett. 336 (1993) 323–326.

[41] H.C. Dietz, R.E. Pyeritz, Mutations in the human gene for fibrillin-1 (FBN1) in the Marfan syndrome and related disorders, Hum. Mol. Genet. 4 (1995) 1799–1809.

[42] A. Piha-Gossack, W. Sossin, D.P. Reinhardt, The evolution of extracellular fibrillins and their functional domains, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0033560.

[43] I. Schrijver, W. Liu, T. Brenn, H. Furthmayr, U. Francke, Cysteine substitutions in epidermal growth factor-like domains of fibrillin-1: distinct effects on biochemical and clinical phenotypes, Am. J. Hum. Genet. 65 (1999) 1007–1020, https://doi.org/10.1086/302582.

[44] M.J. Sherratt, D.V. Bax, S.S. Chaudhry, N. Hodson, J.R. Lu, P. Saravanapavan, C.M. Kielty, Substrate chemistry influences the morphology and biological function of adsorbed extracellular matrix assemblies, Biomaterials 26 (2005) 7192–7206, https://doi.org/10.1016/j.biomaterials.2005.05.010.
[45] D. Gheduzzi, R. Sammarco, D. Quaglino, L. Bercovitch, S. Terry, W. Taylor, I. Ronchetti, Extracutaneous ultrastructural alterations in pseudoxanthoma elasticum, Ultrastruct. Pathol. 27 (2003) 375–384.
[46] D. Quaglino, F. Boraldi, G. Annovi, I. Ronchetti, Multifaceted complexity of genetic diseases: a lesson from Pseudoxanthoma Elasticum, in: K. Ikehara (Ed.), Advances in the Study of Genetic Disorders, Intech Publ, 2011, pp. 289–318, , https://doi.org/10.5772/22161.
[47] M.B. Contri, C. Fornieri, I. Ronchetti, Elastin-proteoglycans association revealed by cytochemical methods, Connect. Tissue Res. 13 (1985) 237–249.
[48] I. Pasquali-Ronchetti, G.M. Bressan, C. Fornieri, M. Baccarani-Contri, I. Castellani, D. Volpin, Elastin fiber-associated glycosaminoglycans in beta-aminopropionitrile-induced lathyrism, Exp. Mol. Pathol. 40 (1984) 235–245, https://doi.org/10.1016/0014-4800(84)90080-7.
[49] I. Pasquali-Ronchetti, D. Quaglino, M. Baccarani-Contri, R. Tenconi, G.M. Bressan, D. Volpin, Aortic elastin abnormalities in osteogenesis imperfecta type II, Coll. Relat. Res. 6 (1986) 409–421, https://doi.org/10.1016/S0174-173X(86)80017-6.
[50] C. Fornieri, M. Baccarani-Contri, D. Quaglino, I. Pasquali-Ronchetti, Lysyl oxidase activity and elastin/glycosaminoglycan interactions in growing chick and rat aortas, J. Cell Biol. 105 (1987) 1463–1469, https://doi.org/10.1083/jcb.105.3.1463.
[51] V.H. Pomin, B. Mulloy, Glycosaminoglycans and proteoglycans, Pharmaceuticals 11 (2018) e27, , https://doi.org/10.3390/ph11010027.
[52] R. Tiozzo Costa, M. Baccarani Contri, M.R. Cingi, I. Pasquali Ronchetti, R. Salvini, S. Rindi, G. De Luca, Pseudoxanthoma elasticum (PXE): ultrastructural and biochemical study on proteoglycan and proteoglycan-associated material produced by skin fibroblasts in vitro, Coll. Relat. Res. 8 (1988) 49–64.
[53] C.M. Kielty, C.A. Shuttleworth, Microfibrillar elements of the dermal matrix, Microsc. Res. Tech. 38 (1997) 413–427, https://doi.org/10.1002/(SICI)1097-0029(19970815)38:4<413::AID-JEMT9>3.0.CO;2-J.
[54] M. Cescon, F. Gattazzo, P. Chen, P. Bonaldo, Collagen VI at a glance, J. Cell Sci. 128 (2015) 3525–3531, https://doi.org/10.1242/jcs.169748.
[55] J.M. Ross, L.V. McIntire, J.L. Moake, H.J. Kuo, R.Q. Qian, R.W. Glanville, E. Schwartz, J.H. Rand, Fibrillin containing elastic microfibrils support platelet adhesion under dynamic shear conditions, Thromb. Haemost. 79 (1998) 155–161, https://doi.org/10.1055/s-0037-1614236.
[56] F. Boraldi, J.S. Burns, A. Bartolomeo, M. Dominici, D. Quaglino, Mineralization by mesenchymal stromal cells is variously modulated depending on commercial platelet lysate preparations, Cytotherapy 20 (2018) 335–342, https://doi.org/10.1016/j.jcyt.2017.11.011.
[57] F. Boraldi, A. Bartolomeo, S. De Biasi, S. Orlando, S. Costa, A. Cossarizza, D. Quaglino, Innovative flow cytometry allows accurate identification of rare circulating cells involved in endothelial dysfunction, PLoS One 11 (2016) e0160153, , https://doi.org/10.1371/journal.pone.0160153.
[58] Y. Kang, S. Kim, A. Khademhosseini, Y. Yang, Creation of bony microenvironment with CaP and cell-derived ECM to enhance human bone-marrow MSC behavior and delivery of BMP-2, Biomaterials 32 (2011) 6119–6130, https://doi.org/10.1016/j.biomaterials.2011.05.015.
[59] D. Hubmacher, S.S. Apte, ADAMTS proteins as modulators of microfibril formation and function, Matrix Biol. 47 (2015) 34–43, https://doi.org/10.1016/j.matbio.2015.05.004.
[60] M. Morikawa, R. Derynck, K. Miyazono, TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology, Cold Spring Harb. Perspect. Biol. 8 (2016) a021873, https://doi.org/10.1101/cshperspect.a021873.
[61] G. Sengle, N.L. Charbonneau, R.N. Ono, T. Sasaki, J. Alvarez, D.R. Keene, H.P. Bächinger, L.Y. Sakai, Targeting of bone morphogenetic protein growth factor complexes to fibrillin, J. Biol. Chem. 283 (2008) 13874–13888, https://doi.org/10.1074/jbc.M707820200.
[62] F. Ramirez, D.B. Rifkin, Extracellular microfibrils: contextual platforms for TGFbeta and BMP signaling, Curr. Opin. Cell Biol. 21 (2009) 616–622, https://doi.org/10.1016/j.ceb.2009.05.005.
[63] R.N. Ono, G. Sengle, N.L. Charbonneau, V. Carlberg, H.P. Bächinger, T. Sasaki, S. Lee-Arteaga, L. Zilberberg, D.B. Rifkin, F. Ramirez, M.L. Chu, L.Y. Sakai, Latent transforming growth factor beta-binding proteins and fibulins compete for fibrillin-1 and exhibit exquisite specificities in binding sites, J. Biol. Chem. 284 (2009) 16872–16881, https://doi.org/10.1074/jbc.M809348200.
[64] J. Taipale, K. Miyazono, C.H. Heldin, J. Keski-Oja, Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein, J. Cell Biol. 124 (1994) 171–181, https://doi.org/10.1083/jcb.124.1.171.
[65] J.P. Annes, Y. Chen, J.S. Munger, D.B. Rifkin, Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1, J. Cell Biol. 165 (2004) 723–734, https://doi.org/10.1083/jcb.200312172.
[66] S.L. Dallas, K. Miyazono, T.M. Skerry, G.R. Mundy, L.F. Bonewald, Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein, J. Cell Biol. 131 (1995) 539–549, https://doi.org/10.1083/jcb.131.2.539.
[67] I.B. Robertson, M. Horiguchi, L. Zilberberg, B. Dabovic, K. Hadjiolova, D.B. Rifkin, Latent TGF-β-binding proteins, Matrix Biol. 47 (2015) 44–53, https://doi.org/10.1016/j.matbio.2015.05.005.
[68] K. Koli, M.J. Ryynänen, J. Keski-Oja, Latent TGF-beta binding proteins (LTBPs)-1 and -3 coordinate proliferation and osteogenic differentiation of human mesenchymal stem cells, Bone 43 (2008) 679–688, https://doi.org/10.1016/j.bone.2008.06.016.
[69] H. Owji, N. Nezafat, M. Negahdaripour, A. Hajiebrahimi, Y. Ghasemi, A comprehensive review of signal peptides: structure, roles, and applications, Eur. J. Cell Biol. 6 (2018) 422–441, https://doi.org/10.1016/j.ejcb.2018.06.003.
[70] Y. Cheng, S. Liu, C. Lu, Q. Wu, S. Li, H. Fu, G. Wang, C. Lv, L. Nie, Y. Zhang, H. Yu, L. Hao, Missense mutations in the signal peptide of the porcine GH gene affect cellular synthesis and secretion, Pituitary 19 (2016) 362–369, https://doi.org/10.1007/s11102-016-0713-6.
[71] R. Mazzieri, V. Jurukovski, H. Obata, J. Sung, A. Platt, E. Annes, N. Karaman-Jurukovska, P.E. Gleizes, D.B. Rifkin, Expression of truncated latent TGF-beta-binding protein modulates TGF-beta signalling, J. Cell Sci. 118 (2005) 2177–2187, https://doi.org/10.1242/jcs.02352.
[72] M.J. Hosen, P.J. Coucke, O. Le Saux, A. De Paepe, O.M. Vanakker, Perturbation of specific pro-mineralizing signalling pathways in human and murine pseudoxanthoma elasticum, Orphanet J. Rare Dis. 9 (2014) 66, https://doi.org/10.1186/1750-1172-9-66.
[73] A.M. Blazquez-Medela, P.J. Guihard, J. Yao, M. Jumabay, A.J. Lusis, K.I. Boström, Y. Yao, ABCC6 deficiency is associated with activation of BMP signalling in liver and kidney, FEBS Open Biol. 5 (2015) 257–263, https://doi.org/10.1016/j.fob.2015.03.009.
[74] G. Schmidmaier, R. Capanna, B. Wildemann, T. Beque, D. Lowenberg, Bone morphogenetic proteins in critical-size bone defects: what are the options? Injury 40 (2009) S39–S43, https://doi.org/10.1016/S0020-1383(09)70010-5.
[75] T. Uchimura, Y. Komatsu, M. Tanaka, K.L. McCann, Y. Mishina, Bmp2 and Bmp4 genetically interact to support multiple aspects of mouse development including functional heart development, Genesis 47 (2009) 374–384, https://doi.org/10.1002/dvg.20511.
[76] J.S. Shao, Z.A. Aly, C.F. Lai, S.L. Cheng, J. Cai, E. Huang, A. Behrmann, D.A. Towler, Vascular Bmp Msx2 Wnt signaling and oxidative stress in arterial calcification, Ann. N. Y. Acad. Sci. 1117 (2007) 40–50, https://doi.org/10.1196/annals.1402.075.
[77] A.F. Zebboudj, M. Imura, K. Bostrom, Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2, J. Biol. Chem. 277 (2002) 4388–4394, https://doi.org/10.1074/jbc.M109683200.
[78] L. Grand Moursel, L.P. Munting, L.M. van der Graaf, S.G. van Duinen, M.T.H. Goumans, U. Ueberham, R. Natté, M.A. van Buchem, W.M.C. van Roon-Mom, L. van der Weerd, TGFβ pathway deregulation and abnormal phospho-SMAD2/3 staining in hereditary cerebral hemorrhage with amyloidosis-Dutch type, Brain Pathol. 28 (2018) 495–506, https://doi.org/10.1111/bpa.12533.
[79] I.A. Radichev, A.G. Remacle, N.E. Sounni, S.A. Shiryaev, D.V. Rozanov, W. Zhu, N.V. Golubkova, T.I. Postnova, V.S. Golubkov, A.Y. Strongin, Biochemical evidence of the interactions of membrane type-1 matrix metalloproteinase (MT1-MMP) with adenine nucleotide translocator (ANT): potential implications linking proteolysis with energy metabolism in cancer cells, Biochem. J. 420 (2009) 37–47, https://doi.org/10.1042/BJ20090082.
[80] C.T. Sigal, M.D. Resh, The ADP/ATP carrier is the 32-kilodalton receptor for an NH2-terminally myristylated src peptide but not for pp60src polypeptide, Mol. Cell. Biol. 13 (1993) 3084–3092, https://doi.org/10.1128/MCB.13.5.3084.
[81] G. Loers, T. Makhina, U. Bork, A. Dörner, M. Schachner, R. Kleene, The interaction between cell adhesion molecule L1, matrix metalloproteinase 14, and adenine nucleotide translocator at the plasma membrane regulates L1-mediated neurite outgrowth of murine cerebellar neurons, J. Neurosci. 32 (2012) 3917–3930, https://doi.org/10.1523/JNEUROSCI.6165-11.2012.
[82] G. Kauffenstein, G.G. Yegutkin, S. Khiati, V. Pomozi, O. Le Saux, G. Leftheriotis, G. Lenaers, D. Henrion, L. Martin, Alteration of extracellular nucleotide metabolism in Pseudoxanthoma Elasticum, J. Invest. Dermatol. 138 (2018) 1862–1870, https://doi.org/10.1016/j.jid.2018.02.023.
[83] V.B. Ayala-Peña, L.A. Scolaro, G.E. Santillán, ATP and UTP stimulate bone morphogenetic protein-2,-4 and -5 gene expression and mineralization by rat primary osteoblasts involving PI3K/AKT pathway, Exp. Cell Res. 319 (2013) 2028–2036, https://doi.org/10.1016/j.yexcr.2013.05.006.
[84] H.S. Kim, J.H. Je, T.G. Son, H.R. Park, S.T. Ji, Y.R. Pokharel, H.M. Jeon, K.W. Kang, H.S. Kang, S.C. Chang, H.S. Kim, H.Y. Chung, J. Lee, The hepatoprotective effects of adenine nucleotide translocator-2 against aging and oxidative stress, Free Radic. Res. 46 (2012) 21–29, https://doi.org/10.3109/10715762.2011.636042.
[85] R. Zarbock, D. Hendig, C. Szliska, K. Kleesiek, C. Götting, Pseudoxanthoma elasticum: genetic variations in antioxidant genes are risk factors for early disease onset, Clin. Chem. 53 (2007) 1734–1740, https://doi.org/10.1373/clinchem.2007.088211.
[86] M. Nemoto, R. Nishimura, T. Sasaki, Y. Hiki, Y. Miyashita, M. Nishioka, K. Fujimoto, T. Sakuma, T. Ohashi, K. Fukuda, Y. Eto, N. Tajima, Genetic association of glutathione peroxidase-1 with coronary artery calcification in type 2 diabetes: a case control study with multi-slice computed tomography, Cardiovasc. Diabetol. 6 (2007) 23, https://doi.org/10.1186/1475-2840-6-23.
[87] L. Duca, S. Blaise, B. Romier, M. Laffargue, S. Gayral, H. El Btaouri, C. Kawecki, A. Guillot, L. Martiny, L. Debelle, P. Maurice, Matrix ageing and vascular impacts: focus on elastin fragmentation, Cardiovasc. Res. 110 (2016) 298–308, https://doi.org/10.1093/cvr/cvw061.
[88] Y. Wang, A.W. Mohsen, S.J. Mihalik, E.S. Goetzman, J. Vockley, Evidence for physical association of mitochondrial fatty acid oxidation and oxidative phosphorylation complexes, J. Biol. Chem. 285 (2010) 29834–29841, https://doi.org/10.1074/jbc.M110.139493.
[89] M. Wajner, A.U. Amaral, Mitochondrial dysfunction in fatty acid oxidation disorders: insights from human and animal studies, Biosci. Rep. 36 (2015) e00281, , https://doi.org/10.1042/BSR20150240.