Original Article

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Rare and intractable fibrodysplasia ossificans progressiva shows different PBMC phenotype possibly modulated by ascorbic acid and propranolol treatment

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SUMMARY Fibrodysplasia Ossificans Progressiva (FOP) is a rare congenital intractable disease associated with a mutation in *ACVR1* gene, characterized by skeleton malformations. Ascorbic acid (AA) and propranolol (PP) in combination is reported to minimize flare-ups in patients. FOP leukocyte phenotype may possibly be modulated by AA and PP treatment. In this study, expression of 22 potential target genes was analyzed by RT-PCR in peripheral blood mononuclear cells culture (PBMC) from FOP patients and controls to determine effectiveness of the combination therapy. PBMC were treated with AA, PP and AA+PP combination. Basal expression of 12 of the 22 genes in FOP PBMC was statistically different from controls. *ACVR1*, *ADCY2*, *ADCY9* and *COL3* were downregulated while *COL1* was upregulated. *ADRB1*, *ADRB2*, *RUNX2*, *TNF-α* and *ACTB*, were all overexpressed in FOP PBMC. In control, AA upregulated *COL1, SVCT1, ACTB, AGTR2* and downregulated *ADCY2*. In FOP cells, AA upregulated *ACVR1, BMP4, COL1, COL3, TNF-α*, *ADCY2, ADCY9, AGTR2* and *MAS*, while downregulated *ADBR2, RUNX2, ADCY1, SVCT1* and *ACTB*. PP increased *ADBR1* and decreased *RUNX2, TNF-α, AGTR1, ACTB* and *CHRNA7* genes in treated control PBMC compared to untreated. PP upregulated *ADBR1, ADBR2* and *MAS*, and downregulated *TNF-α* and *ACTB* in treated FOP PBMC versus untreated. AA+PP augmented *ADRB1* and *ADRB2* expressions in control PBMC. In FOP PBMC, AA+PP augmented *ACVR1, COL1, COL3, ADBR1, AGTR2* and *MAS* expression and downregulated *ADBR2, RUNX2, ACTB* and *MRGD*. These data show distinct gene expression modulation in leukocytes from FOP patients when treated with AA and or PP.

Keywords FOP, gene expression modulation, peripheral blood mononuclear cells, FOPCON

1. Introduction

Fibrodysplasia ossificans progressiva (FOP) is a rare intractable autosomal dominant disease affecting one in every two million individuals, characterized by congenital skeletal malformations and postnatal heterotopic ossification. In newborns FOP does not stimulate developmental skeletal deformation, except for hallux valgus (*1*). Classical FOP individuals have a heterozygous mutation (c.617G>A, p.R206H) in the *ACVR1* receptor gene, or *ALK2*, located on chromosome 2q23-24 (*2*). This mutation confers a gain of function, activating the signaling pathway of BMP [bone morphogenic protein] independent of ligand stimulation and also functions as a Type II receptor BMP independent. In addition, activin-A, a TGF-β-related cytokine, and BMP, competitive antagonist in wild *ACVR1*, is recognized as an agonist in *ACVR1* R206H (*1,3*).

FOP pathophysiology shows an impaired BMP signaling pathway that correlates to ontogeny defects in embryonic stage and to development and progress

of heterotopic ossification (HO) in postnatal life. This is often preceded by inflammatory processes induced or spontaneous (*1,2*), favored by a less perfused and acidic pH tissue microenvironment. Further, this aberrant process usually begins in the first decade of life and progresses with developmental maturation, leading to ankylosis of the major joints and chest fusion. Immune system neutrophils, macrophages and importantly mast cells are recruited, stimulating secretion of several cytokines. Muscle tissue and adjacent soft tissues are degraded and replaced by fibroproliferative cells that generate cartilage and subsequently ectopic bone (*4*).

Pathways of the autonomous nervous system (ANS) play important roles in neovascularization and in final osteoblast and osteoclasts differentiation (*5*). Gaps exist in knowledge of the role of adrenergic pathways and receptors in the algesia, inflammatory crises and in ectopic bone formation in FOP. Understanding of neural anti-inflammatory pathways functioning as important neuronal regulators of immune response need to be clarified (*6*). Imbalance of the two main axes of the renin-angiotensin system (RAS) has also been implicated in the pathogenesis of this and other inflammatory and fibrotic processes (*7*).

Curative therapy is currently not available, nor FOP medications completely free of side effects. FOP management aims to control flare-ups and symptoms by corticosteroids, mast cell inhibitors, non-steroidal anti-inflammatory drugs, cyclooxygenase inhibitors, bisphosphonates, muscle relaxants, bone marrow transplants, rosiglitazone, retinoic acid receptor agonists and commonly used treatments for pain, including narcotic analgesics (*1*). Clinical trials are being developed with anti-activin A antibodies/REGN2477 (phase 2), Rapamycin (phases 2, 3) and Palovarotene (phase 3). Results are promising, with adverse effects under study and carefully monitored (*3,8*). A wide range of molecular mechanisms is involved in the exacerbated activation of the mutated gene in FOP in addition to the aberrant BMP-Smad 1/5/8 signaling (*9*). It is relevant to consider the study of factors not yet explored in FOP.

In some patients, FOP symptoms are improved by ascorbic acid (AA). This antioxidant, anti-inflammatory and modulator of collagen synthesis is reported to reduce outbreaks, and transiently stabilize crisis, either used alone or in combination with disodium etidronate (*10*). Long studied and approved for various clinical indications, ascorbic acid is a therapeutic possibility for anti-growth and invasiveness of solid cancers, and is a useful therapeutic supplement in several angiogenic diseases (*11,12*). Despite many studies demonstrating the efficacy of AA in gene expression regulation, and genomic modulation and differentiation of embryonic stem cells (*11-14*), it has not been considered for regulation of FOP pathophysiology pathways.

Since AA may help in FOP treatment, and the nonspecific adrenergic β-blocker propranolol (PP) has surfaced as an important treatment of infantile hemangioma with potential antiangiogenic effects (*15*), Palhares *et al.* (*10*), have suggested using propranolol with ascorbic acid (FOPCON) for continuous administration to FOP patients. However, the mechanisms by which AA+PP work are not yet clarified. The hypothesis of the work presented here is that transcription of genes directly or indirectly involved in heterotopic ossification is modulated by vitamin c and the β-blocker propranolol in a cell culture model of PBMC from FOP patients, compared to control individuals.

2. Materials and Methods

2.1. Samples

Peripheral blood mononuclear cells (PBMC) were cultured using peripheral whole blood samples collected by antecubital venipuncture from volunteers (FOP [n = 8] and healthy control subjects [n = 8]). Whole blood was collected in heparinized tubes, kept at 4°C for up to 24 hours prior to processing. Volunteer participants were informed and signed the consent form (ICF). This research was approved by Research Ethics Committee of the Federal University of Minas Gerais (document #403073/CAAE 17422113.3.0000.5149).

2.2. PBMC culture

PBMC method was performed as previously described (*16*) with some modification. Briefly, 15 mL heparinized blood was transferred to 50 mL Falcon tube containing Ficoll-diatrozoate mixture (Histopaque®-1077, Sigma® 10771), ratio 1:2 of Ficoll-diatrozoate/blood. The leukocyte ring obtained by ficoll density gradient by centrifugation 40 min/1.400 rpm/24°C, maximum acceleration specification and minimum braking at high-speed centrifuge (Heraeus Multifuge X3R Centrifuge - Thermo SCIENTIFIC®). Mononuclear cells were collected from plasma:Ficoll-diatrozoate interface, transferred to new tubes and washed 3× in culture medium, twice in sterile DEMEM medium (Gibco® pH, 7.2 to 7.4) and once in complete RPMI 1640 medium (10% fetal bovine serum + L-glutamine + gentamicin + streptomycin, pH 7.4) (Gibco® pH, 7.2 to 7.4). Cells washed at 1200rpm/7 min/4°C were resuspended in 1 mL complete RPMI Medium. Cell density was adjusted to desired concentration after counting in Newbauer Chamber with Trypan Blue. Required sterile procedures were performed in laminar flow hood (BIOSEG® 12, VECO Group). Cell culture was performed in a 24-well plate, with 640 or 800 microliters of the cell suspension (1.2×10^6 or 1.5×10^6 cells) cultured and stabilized for 24 hours in complete RPMI 1640 medium, maintained in a CO_2 incubator (5%) at 37°C (Thermo scientific® / Forma Series II Water Jacket CO_2 incubator).

2.3. Treatments of PBMC

Treatment with propranolol, ascorbic acid and propranolol plus ascorbic acid was performed after 24 hours of cell cultivation and stabilization, in triplicate for 96-well plate cell viability assessment at 3×10^5 cells per well and in 24-well plates, for 24 hours in CO_2, incubator at 1.2×10^6 or 1.5×10^6 cells for Real-time PCR. Ascorbic acid (L [+] - Ascorbinsaure Zur Analyze; Vitamin C C6H8O6 pro Analysis 13496OS Art. 127 pa Merck®) dosage used for the treatment was standardized, and optimal dosage (2 mM) was maintained (*16*). Propranolol (Propranolol HCL from CHANGZHOU YABANG® evaluated Quality Control by All Chemistry Laboratory under number ALL 46092-1) treatment dose was 15 µM (*17,18*). Ascorbic acid solution was prepared in complete RPMI 1640 Medium (AA, q.s.p 5 mL of medium), and pH adjusted to 7.4 with NaOH. Propranolol was also prepared in complete RPMI 1640 Medium (0.22 mg propranolol, q.s.p. 5 mL medium) but no pH adjustment was required. Both solutions were sterile filtered (SF; 22 µm) under laminar flow hood (BIOSEG® 12, VECO Group). Trypan blue stained cells were counted after 24 hours of treatment to analyze viability.

2.4. Total RNA extraction

Cultured 1.2×10^6 to 1.5×10^6 treated and untreated cells were transferred from plate to 1.5 mL microcentrifuge tubes, centrifuged immediately at 1,200 rpm (bench-top refrigerated centrifuge, 3K30 Sigma®) for 7 min, to concentrate cell pellet. Total RNA was extracted using Stat-60® reagent, according to manufacturer protocol. RNA was resuspended in 25 µL of DEPC water, quantified at 260 nm in a Denovix® DS-11 nanodrop. Total RNA was DNase I treated (TURBO DNA-free kit, Ambion Inc., Foster, California, USA), DNase I was inactivated with EDTA/75ºC/10 min according to manufacturer's protocol. Aliquots were re-quantified at 260 nm for later use in RT-PCR.

2.5. Oligonucleotide primers

Oligonucleotide primers, described in Table 1, for reverse transcription (RT) and real-time PCR (qPCR) were designed through GenBank sequences in BLASTn program analysis (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*), synthesized by IDT (Integrated DNA technologies; *http://www.idtdna.com*), received lyophilized, resuspended in sterile filtered H_2O (0.22 µm; q.s.p. 100 pmol/µL) and stored as 10 pmol/µL at -20°C. S26 mRNA was the endogenous normalizer.

2.6. Reverse transcription (RT) and Real-time PCR (qPCR)

Single-stranded complementary DNA synthesis (sscDNA) was performed by RT. Briefly, 700 ng of RNA was pre-incubated at 70ºC for 10 min with 10 pmol of each reverse primer with 10 pmol of oligo dT18 primer (Invitrogen), followed by ice storage on the bench.

Table 1. Targeted genes and selected oligonucleotide primers

Gene	mRNA Description	Oligonucleotide primer sequences	Target (bp)
ACVR1	activin A receptor type 1	F<CTGCCTTCGAATAGTGCTGTCCAT>R<TAAATCTCGATGGGCAATGGCTGG	100
BMP4	bone morphogenetic protein 4	F<CAGGAGATGGTAGTAGAGGGATGT>R<AGTCTGTGTAGTGTGTGGGTGA	140
COL1	collagen type I α-1 chain	F<CAAAGGAGACACTGGTGCTAAG>R<CTCCTCGCTTTCCTTCCTCTC	89
COL3	collagen type III α-1 chain	F<AGCTGTTGAAGGAGGATGTTCCCA>R<TTTGGCATGGTTCTGGCTTCCA	77
ADRB1	Adrenoceptor β-1	F<TTCTACGTGCCCCTGTGCATC>R<GATCTTCTTCACCTGCTTCTGG	78
ADRB2	adrenoceptor β-2	F<CTGTGCGTGATCGCAGTGGAT>R<CTTATTCTTGGTCAGGCTC	78
RUNX2	RUNX family trans. factor 2	F<CTTGACCATAACCGTCTTCAC>R<CGAGGTCCATCTACTGTAACT	81
TNF-α	tumor necrosis factor α	F<CCAGGGACCTCTCTCTAATCA>R<CTTTGCTACAACATGGGCTAC	95
ACTB	β-actin mRNA	F<TCACCCACACTGTGCCCATCTACGA>R<CAGCGGAACCGCTCATTGCCAATGG	295
ADCY1	adenylate cyclase 1	F<TGGTCACCTTCGTGTCCTATG>R<CTGTGACCAGCAAGTGCGACG	98
ADCY2	adenylate cyclase 2	F<GCCTTGTTGCCATGGGATACCT>R<TGAAGAGGAAGAACGATACCTG	81
ADCY7	adenylate cyclase 7	F<GTGTTCGACGCATGGACAAAG>R<GCTGAAGGGCAGTAGTGTGTA	96
ADCY9	adenylate cyclase 9	F<GCTACCGGGTCCTCAACGAG>R<ATGTACGTGGCTCCGATGGT	103
SVCT1	Sodium vit. C carrier type 1	F<ACTCTCCTCCGCATCCAGATCTTC>R<TGTCAAGGTCAGGACATAGCA	90
SVCT2	Sodium vit. C carrier type 2	F<TGCTCGAGCCATCCTGTCTTTAG>R<AGATGTGTTCTGTGTGCAACAG	98
AGTR1	angiotensin II receptor type 1	F<TTCAGCCAGCGTCAGTTTCA>R<GGCGGGACTTCATTGGGT	101
AGTR2	angiotensin II receptor type 2	F<TATGGCCTGTTTGTCCTCATTG>R<CCATTGGGCATATTTCTCAGGT	115
MAS	MAS1 proto-oncogene GPCR	F<GCTACAACACGGGCCTCTATCTG>R<TACTCCATGGTGGTCACCAAGC	160
MRGD	MAS related GPCR D	F<TCCCTGCCTCTGAGCATCTA>R<GAGAGGCGTGACAAGCTGAA	100
CHRNA7	Cholinergic α-7 subunit receptor	F<CTTTACAAGGAGCTGGTCAAGAAC>R<GCTCAGGGAGAAGTAGACGGTGA	90
IL10	interleukin 10	F<ATGAGCATTCAGACTGGGTAAAC>R<TTTTAGGGGCTAAGAAACGCAT	123
ALPL	alkaline phosphatase	F<TGTCATCATGTTCCTGGGAGATGG>R<CAGGGTTGTGGTGGAGCTGAC	86
S26	S26 ribosomal protein RNA	F<TGTGCTTCCCAAGCTGTATGTGAA>R<CGATTCCTGACTACTTTGCTGTG	75

(Bp) base pair; (F) forward sense (5′-3′); (R) reverse anti-sense (5′-3′).

Then, 40 U (11 µL) of reverse transcriptase enzyme mix in RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) containing 2 µL of dNTP mix (10 mM each) were incubated at 45°C/1 hour, with RNA and primer solution. RT was terminated at 4°C and immediately used in qPCR, or frozen at -20°C, until qPCR. All reagents were from Invitrogen™ (SuperScript™ First-Strand Synthesis System for RT-PCR). sscDNA samples were used in qPCR performed on *QuantStudio 6 Flex Real-Time System*® (ThermoFisher Scientific,) using reaction protocol described by the SYBR Green PCR Master Mix Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Triplicate samples were applied to 384-well plates (ABI PRISM® 384-Well Optical Reaction Plate with Barcode, Invitrogen Life Technologies, Carlsbad, CA, USA), in a final reaction volume of 10 µL each. Aliquots of 0.8 µL of sscDNA from the samples were pipetted into each channel of the plate plus 9.2 µL of SYBR Mix (5 µL of the SYBR Green PCR Master Mix Kit, 0.6 µL of each primer (sense and antisense; 10 pmol/µL) and 3 µL sterile filtered water). The plate was sealed with optical adhesive (ABI PRISM® Optical Adhesive Covers, Invitrogen Life Technologies, Carlsbad, CA, USA). qPCR performed as: [stage 1] a 50°C / 2 min cycle; [stage 2] a cycle at 95°C/10 min; [stage 3] 40 cycles of 95°C/15 s, followed by a dissociation curve from 60°C.

Relative quantification of mRNA expressions determined by comparative analysis with endogenous control, using comparative CT method, as $2^{-\Delta\Delta CT}$ method for relative levels of gene expression was applied (*19*). Data were analyzed in *GraphPad Prism 5* program for statistics, and unpaired *t* test plus ANOVA were applied. Results were statistically significant for $p \leq 0.05$.

3. Results

3.1. PBMC viability

Viability was assessed by trypan blue staining. PBMC were viable after treatment with Propranolol and ascorbic acid at 15 µM and 2 mM, respectively, and used in experiments.

3.2. Phenotype profile

Phenotype profile differences were detected as observed (Figure 1, Table 2, Tables S2 and S4, http://*www.irdrjournal.com/action/getSupplementalData.php?ID=74*) by the variations in mRNA expression among PBMC of control individuals versus FOP PBMC. Twelve out of 22 genes (54.5%) showed significant expression differences, when baseline mRNA expression was compared to control cells. There was no significant difference of baseline mRNA expression for *BMP-4*, *ADCY7*, *SVCT2*, *AGTR1*, *AGTR2*, *MAS*, *MRGD*, *CHRNA7*, *IL-10* and *ALPL* genes (Figure 1, Table 2).

3.3. Ascorbic acid effect on gene expression

Expression data demonstrated gene modulation by AA in both patient and control PBMC in culture (Table 2 and Table S1, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*). AA treatment of normal PBMC modulated five out of 22 genes (22.7%), with *COL1*, *ACTB*, *SVCT1* and *AGTR2* upregulated and *ADCY2* downregulated, while significant upregulation of *ACVR1*, *BMP4*, *COL1*, *COL3*, *ADRB1*, *TNF-α*, *AGTR2* and *MAS* occurred in FOP PBMC. When AA treated FOP PBMC was compared to untreated FOP PBMC, there was downregulation of *ADRB2*, *RUNX2*, *ACTB* and *ADCY1* genes (Table 2 and Table S3, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*). Further, downregulation was observed in *MAS* and *MRGD* genes, in controls (Tables 2 and Table S1, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*), at both baseline and after treatment. *ADCY1*, *2*, *7* and *9*, *SVCT1* and *SVCT2* coding genes, were here checked only for AA. All *ADCY*, but 7, were altered in FOP PBMC

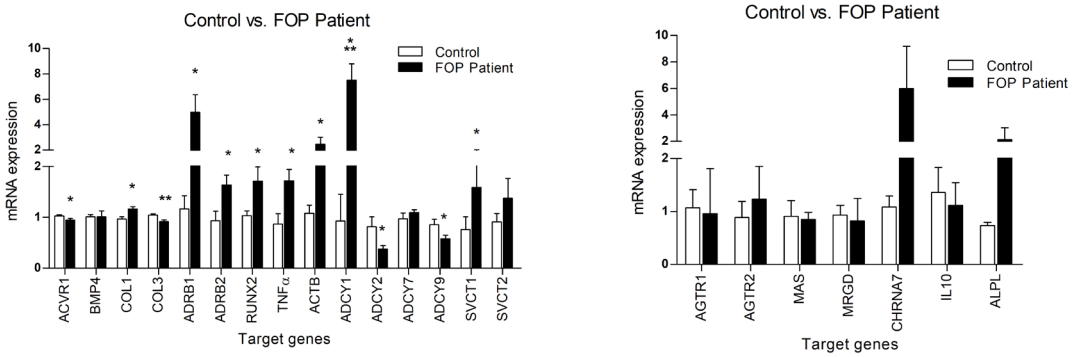

Figure 1. Distinct gene expression profiles between FOP peripheral blood mononuclear cells versus control cells. FOP PBMC basal profile gene expressions were statistically different compared to controls ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). *ACVR1*, *ADCY2*, *ADCY9* and *COL3* showed downregulated and *COL1* upregulated. *ADRB1* and *2*, *RUNX2*, *TNF-α* and *ACTB*, were most overexpressed in FOP PBMC among evaluated mRNAs. There was no significant difference of baseline mRNA expression for *BMP-4*, *ADCY7*, *SVCT2*, *AGTR1*, *AGTR2*, *MAS*, *MRGD*, *CHRNA7*, *IL-10* and *ALPL* genes.

Table 2. Illustration of gene modulation in FOP PBMC and groups control in response to *in vitro* treatments with ascorbic acid (AA), propranolol (PP) and propranolol combined with ascorbic acid (PPAA) and comparisons

Genes	FOP X Control	Cont. AA X Cont.	FOP AA X FOP	FOP AA X Contr.	FOP AA X Contr. AA	Contr. PP X Contr.	FOP PP X FOP	FOP PP X Contr.	FOP PP X Contr. PP	Contr. PPAA X Contr.	FOP PPAA X FOP	FOP PPAA X Contr.	FOP PPAA X Contr. PPAA
ACVR1	↓	-	↑	↑↑	↑↑	-	-	-	-	-	↑	↑	↑↑
BMP4	-	-	↑↑	↑	↑↑	-	-	-	↑	-	-	-	-
COL1	↑	↑	↑↑	↑↑↑	↑↑	-	-	-	-	-	↑	↑↑	-
COL3	↓↓	-	↑	↑↑	↑↑	-	-	-	-	-	↑	↑	-
ADRB1	↑	-	↑	↑	↑	↑	↑	↑↑	↑	↑↑	↑	↑	-
ADRB2	↑	-	↓	-	↓	-	↑	↑	↑	↑	↓	-	-
RUNX2	↑	-	↓	-	↓	↓	-	-	↑	-	↓	-	-
TNF-α	↑	-	↑	↑	↑	↓	↓	-	↑	-	-	↑	↑
ACTB	↑	↑	↓	-	↓	↓↓	↓↓	↓↓	-	-	↓	-	↓
ADCY1	↑↑↑	-	↓↓	-	N	N	N	N	N	N	N	N	N
ADCY2	↓	↓	↑	-	N	N	N	N	N	N	N	N	N
ADCY7	-	-	-	-	N	N	N	N	N	N	N	N	N
ADCY9	↓	-	↑↑↑	-	N	N	N	N	N	N	N	N	N
SVCT1	↑	↑	↓	-	N	N	N	N	N	N	N	N	N
SVCT2	-	-	-	-	N	N	N	N	N	N	N	N	N
AGTR1	-	-	-	-	-	↓	-	↓	-	-	-	-	↓
AGTR2	-	↑	↑↑	↑↑↑	-	-	-	↑	↑	-	↑	↑	↑
MAS	-	-	↑	↑	↑	-	↑	↑	↑	-	↑	↑↑	↑
MRGD	-	-	-	↓↓	↓↓	-	-	↓↓	↓	-	-	↓↓	↓↓↓
CHRNA7	-	-	-	↓↓	↓↓	↓	-	-	-	-	-	↓↓	↓
IL-10	-	-	-	-	↓	-	-	-	-	-	-	-	-
ALPL	-	-	-	↑	-	-	-	-	-	-	-	-	-

Modulation (arrows); (-) not significant; ↑/↓ = up/downregulation ($p \leq 0,05$); ↑↑/↓↓ = up/downregulation ($p = 0,001$ to $\leq 0,01$); ↑↑↑/↓↓↓ = up/downregulation ($p < 0,001$); (N) not experimented.

compared to normal PBMC base line expressions. When FOP PBMC were treated with AA, *ADCY 1*, *2* and *9* were reversed, that is, upregulated *ADCY 1* showed a reduction in expression, while downregulated *9* and *2*, were increased. However, the final expression of all *ADCY* analyzed were brought to physiological mRNA expression levels, similar to control PBMC (Table 2).

3.4. Propranolol effects

In PBMC of normal individuals PP modulated 37.5% (6/16) of genes. It increased *ADBR1* and decreased *RUNX2*, *TNF-α*, *AGTR1*, *ACTB* and *CHRNA7* genes in control PBMC, compared to untreated control (Table 2). FOP PBMC responded to PP by modulating 31.2% (5/16) genes by increasing *ADRB1*, *ADRB2* and *MAS*, while decreasing *TNF-α* and *ACTB* when compared to baselines of untreated FOP PBMC (Table 2 and Table S3, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*). PP treated FOP PBMC compared to untreated control PBMC, kept increased *ADBR1* and *ADBR2*, while showing significant downregulation of *AGTR1*, *MRGD* and *CHRNA7*, and upregulation of *AGTR2* and *MAS* in FOP cells in response to treatment with propranolol (Table 2 and Table S2, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*).

3.5. Ascorbic acid and propranolol (AA+PP) combination effects on gene expression

In normal control PBMC, the combination of AA with PP resulted in the modulation of only two out of 16 genes (12.5%) studied, up regulation of *ADBR1* and *ADBR2* (Table 2 and Table S1, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*). However, the effect of AA+PP over PBMC of FOP carriers, compared to normal control PBMC, resulted in a statistically significant modulation of FOP gene profile, by upregulating *ACVR1*, increasing *COL1*, reversing *COL3* from down to upregulation, kept same profile for *ADRB1* but normalized *ADBR2*, counter-regulatory modulations in the expression of *ACVR1*, *COL3*, *ADRB2*, *RUNX2* and *ACTB* in relation to the baseline state of FOP PBMC, while increasing *AGTR2* and *MAS* genes, AA+PP downregulated *ADRB2*, *RUNX2* and normalized *ACTB* (Table 2 and Table S2, *www.irdrjournal.com/action/getSupplementalData.php?ID=74*). *MRGD* and *CHRNA7* mRNA expressions were significantly downregulated by AA+PP, when FOP PBMC were compared to normal control group and after treatment. An overview of summarized data is shown in Table 2 and Figure 2.

4. Discussion

The study of FOP is hindered by tissue sample restrictions inherent to deep connective tissues traumas which trigger HO. Cellular models, including Epstein-Barr transformed lymphoblast cell lines, dental pulp

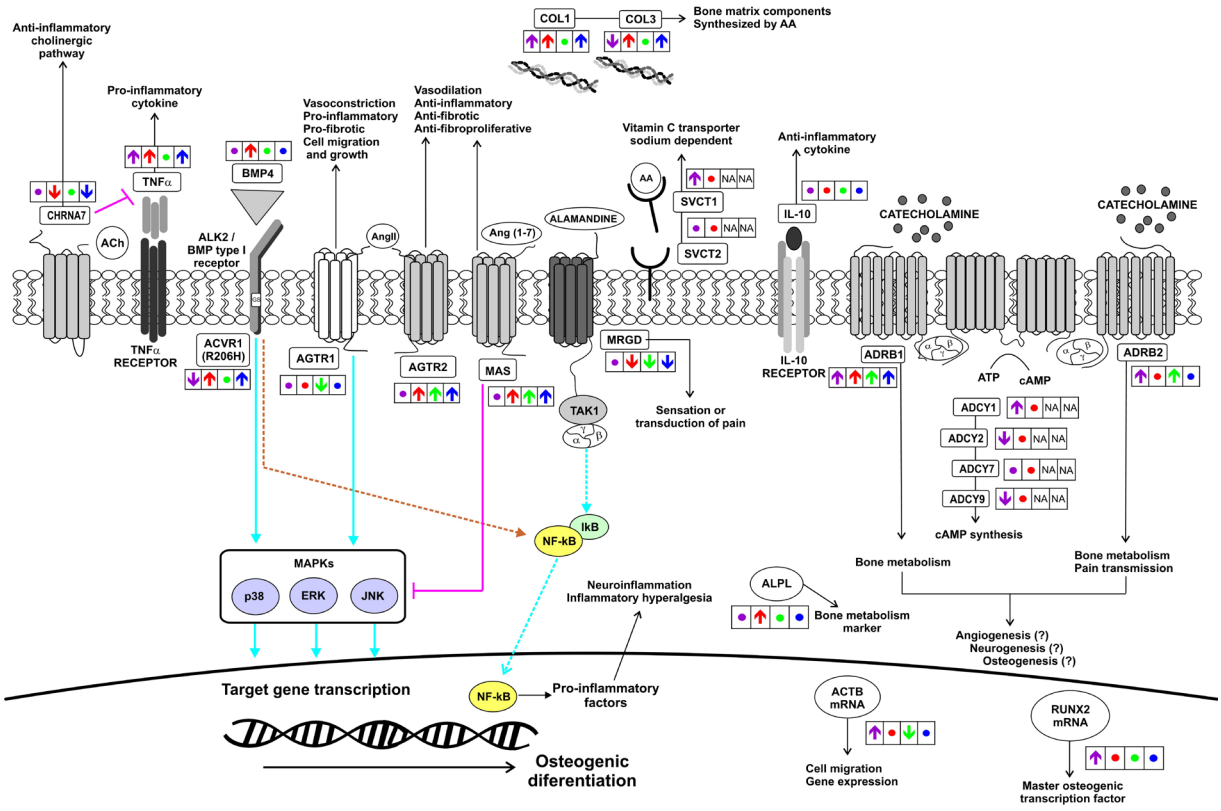

Figure 2. Proposed mode of ascorbic acid + propranolol effect on the expression of downstream targets modulating fibrodysplasia ossificans progressiva peripheral blood mononuclear cells compared to normal PBMC. FOP PBMC mRNA expressions compared to normal control PBMC at basal (**Purple**), AA treatment (**Red**), PP treatment (**Green**) and AA+PP treatment (**Blue**); (●) equal to control or unchanged; (**NA**) not experimented. FOP PBMC basal mRNA expression was statistically significant ($p \leq 0.05$) (Figure 1) when compared to controls. *ACVR1*, *ADCY2*, *ADCY9* and *COL3* showed downregulated and *COL1* upregulated. AA upregulated *ACVR1*, *BMP4*, *COL1*, *COL3*, *TNF-α*, *ADCY2*, *ADCY9*, *AGTR2* and *MAS*, while downregulating *ADBR2*, *RUNX2*, *ADCY1*, *SVCT1* and *ACTB*. PP upregulated *ADBR1*, *ADBR2* and *MAS*, while downregulating *TNF-α* and *ACTB*. The combination of AA+PP augmented expressions of *ACVR1*, *COL1*, *COL3*, *ADBR1*, *AGTR2* and *MAS* but downregulated *ADBR2*, *RUNX2*, *ACTB* and *MRGD* genes, when compared to untreated (OBS: the graph map molecules are only representative designs, but without complex structural biochemical purpose).

stem cells from FOP children (*20*), and induced stem cells from dermal fibroblasts from skin biopsy (*21*), are important tools, yet of limited access. We evaluated cultured FOP PBMC and data points to a prone unbalanced inflammatory phenotypic profile state of gene expressions in FOP cells. FOP cells showed an altered basal gene expression profile. Renin-angiotensin system receptor genes did not comprise the mRNA profile of FOP, yet they were clearly regulated in response to proposed treatments. AA and PP alone or in combination, are shown to modulate anti-inflammatory gene effects. Dose results used were consistent with literature of different cell cultures (*13,17,18,22*).

Half of the genes evaluated from FOP PBMC were sensitive to treatments, in contrast to ~13% of genes in control PBMC. PP alone modulated eight genes in FOP PBMC and six in normal PBMC, among 16 genes studied. AA and PP combination changed expressions of ~63% of FOP PBMC analyzed genes, while only two genes were regulated in the control group. These data show that the PBMC model may useful while elucidating the broader impact of the *ACVR1* gene mutation in concert with the other FOP-related genes not yet included in pathophysiology pathways. AA may modulate genes epigenetically as verified in other studies (*13,23*).

There are few reports on AA+PP pharmacokinetics interactions. AA may influence PP absorption and first-pass metabolism in healthy young humans, slightly reducing plasma PP availability and decreasing its urinary excretion, but elimination rate is not changed (*24*). Apart from this, AA+PP may contribute to the beneficial effects of beta-blockers that minimize human atrial fibrillation (*25*). The interaction of AA+PP *in vivo* as well as *in vitro* remains to be clarified. AA shows Na^+ dependent high affinity to transporters SVCT1 and SVCT2 proteins after digestion. SVCT1 expression has been shown to occur in intestine and kidney, transporting AA into and from the blood. However, it remains unclear if SVCT1 is an AA receptor and therefore how AA is transported in pathological conditions (*13*). Membrane bound SVCT2 allows intracellular AA to exceed

extracellular concentration (*13,23*). Our data showed *SVCT2* gene expression is stable, with no alteration of expression in both FOP and control cells. Yet, *SVCT1* was overexpressed in FOP PBMC and sensitive to the AA downregulation effect in FOP PBMC.

PBMC adenylate cyclase coding genes *(ADCY) 1*, *2*, *7* and *9*, were also checked for AA modulation. *ADCY 1*, *2* and *9* expressions were altered in FOP PBMC, yet AA reversed this and brings expression back to physiological levels. Additionally, using Northern blot and qPCR analysis, others have reported *ADCY1*, *ADCY7* and *ADCY9* are highly expressed while *ADCY2* is downregulated in peripheral blood leukocyte cells (*26*). AA modulation of *ADCY* genes shows a new approach to target pathophysiology of FOP. Given that AA is a competitive inhibitor of ADCY, it may suppress genes under control of cAMP-dependent pathways (*13*), changing intracellular cAMP concentrations, thus inhibiting peripheral myelin protein-22 (PMP22) by suppressing *PMP22* gene expression (*13,27*). Intracellular cAMP favors ubiquitous expression of proinflammatory mediators such as TNFα and IL-10 (*26,28*). AA's role in modulation of ADCY genes demonstrates the importance of specific receptors coupled to heterotrimeric G proteins (*29*). The abnormal *ADCY* profile in FOP PBMC may be involved in the molecular unbalance of FOP and *ADCY7* is likely linked to this disorder, though expressed, was not modulated or impacted by AA.

FOP involves complex pathophysiological pathways in which signaling and response of immunoinflammatory factors differ greatly from normal defensive inflammatory mechanisms. ADRB1 and ADRB2, and possibly others receptors, seem to participate in the sympathetic regulation of HO stages, such as angiogenesis, neurogenesis and osteogenesis (*5,6*). Reports demonstrate catecholamines and additional signaling cascades of the sympathetic nervous system (SNS) and immune system interact through cytokine production in lymphocytes, dependent on β2 adrenergic receptors density in PBMC (*30*). A cause-effect relationship of ADCY system dysregulation and β-adrenergic receptors downregulation in lymphocytes suggests impairment of β-adrenergic transmembrane signaling in septic patients, linking ADCY to β-adrenergic pathways (*31*). In this regard, it is noteworthy to highlight the fact that unspecific adrenoreceptor antagonists are not well studied in FOP.

Togari (*32*) studying bone resorption processes, observed SNS modulation of osteoclast differentiation and osteoclastogenesis inhibiting factors produced by osteoblast/stromal cells with adrenergic and neuropeptide receptors. Furthermore, deletion of *ADRB1*, *2*, or both, leads to altered bone phenotypes. While ADRB1 signaling is shown to regulate anabolic bone responses, ADRB2 regulates bone remodeling through the expression of tumor necrosis factor TNFSF11(RANKL) in osteoblasts (*33*). The role of PBMC signaling in HO is not clear. Genes *ADRB1* and *2* were overexpressed in FOP PBMC before treatment. AA+PP downregulated *ADRB2*, suggesting *ADRB2* receptor as putative candidate in a FOP pathophysiological pathway and its response to AA+PP may benefit FOP as suggested by Palhares *et al.* (*10*). These results may provide possible routes to be explored in pharmacotherapy studies of FOP and HO. There is evidence that ADRB are expressed in human macrophages and monocytes to generate anti- and pro-inflammatory effects, hinged on how they are activated or inhibited, possibly showing that receptor responsiveness changes during cell differentiation (*34*).

Post-translationally modified type III pre-procollagen is a main component of bone matrix, contributing to proper maintenance, physiology, and coordination of post-injury repair; all processes dependent on L-ascorbic acid (*12,35*) and is a regulator of type I and II collagen fibril diameter (*36*). AA stimulates the synthesis of types I and III collagen in fibroblasts *in vitro*, where it stabilizes and upregulates its mRNA expression, without altering the cellular protein presentations (*37*). AA treatment of FOP (*38*) was originally based on the hypothesis that AA possibly modulates collagen gene expression and deposition (*13,38*). *COL3* downregulation in FOP PBMC may be fundamental in FOP pathophysiology. Low *COL3* could lead to weakened endochondral tissue, facilitating infiltration and establishment of local inflammatory processes during flare-ups. AA or AA+PP *COL3* upregulation may improve tissue resistance by favoring anti-inflammatory environment. Additionally, AA positive influence on type-I and -III collagen synthesis could contribute to a reduction in new bone deposition based on angiostatic effects (*12,39,40*). Nevertheless, overexpression of *COL1* found in FOP PBMC should be further investigated, in view that type I collagen largely coats some blood vessels in developing bone, possibly secreted from osteoblasts and endothelial cells (*41*), noting that PP alone did not show effects on the collagen mRNA.

ALPL (Alkaline Phosphatase, Liver/Bone/Kidney) activity in muscle satellite cells is induced by ACVR1 (R206H), inhibiting antagonists and increasing BMP4 for osteoblasts formation (*2,42*). FOP patients may show increased serum ALPL, especially in flare-ups (*43*), however, FOP PBMC *in vitro* showed no *ALPL* expression differences when compared to PBMC controls in stabilized cultures. It seems, though, that FOP *ALPL* increases seen *in vivo* depend on multifactorial compounds for final HO. The RUNX2 transcription factor, a major regulator of osteoblast differentiation *via* SMAD1 signaling and involved in the final ossification process, requires local BMP production. The combined expression of BMPs and RUNX2 stimulates osteoblastic gene expression in FOP primary teeth isolated cells (SHED). These SHED have been shown to mineralizes faster than control cells with high expression of *ALPL* (*20*). In our study FOP PBMC showed increased *RUNX2* expression.

Treatment with AA or AA+PP decreased *RUNX2* expression levels while not altering expression of *ALPL*. Recent studies have shown AA dose-dependent modulation of osteogenic gene expression in human osteosarcoma G292 cells and high doses of AA leads to downregulation of *RUNX2* and *ALPL* expression (*44*). This suggests that AA treatment may inhibit osteoblast maturation. High doses of AA can act as a pro-oxidant that drives ALPL activity increases after osteogenic induction by BMP2 facilitated by oxidative stress (*10,44*). Indeed, downregulation of *RUNX2* may benefit FOP clinical conditions by minimizing HO.

Despite the striking flare-up process preceding HO, targeted studies on inflammatory genes in FOP are crucial, for example, TNF-α role in HO is paradoxical. The transient inhibition of RUNX2 function during skeletogenesis, due to the activity of Twist proteins (-1 and -2), whose gene expression is induced by TNF-α (*45*), needs to be clarified. For instance in *Nfactc1-Cre/caAcvr1fl/wt* mice, a genetic model similar to FOP, TNF-α serum levels are elevated and also histologically located in HO anlagen cartilaginous formation areas (*46*). However, studies exploring cytokine modulation of FOP demonstrate that plasma TNF-α levels are above average in patients undergoing flare-up (*4*), while IL-10 plasma levels are significantly increased in FOP subjects with no flare-up (*47*).

AA supplementation modulated various genes in a PBMC microarray study, from healthy individuals, mainly under inflammatory stimulation by LPS. TNF-α and pro-inflammatory cytokines were activated and released in fresh PBMC before and after AA supplementation, but IL-10 was released only after supplementation (*48*). Similarly, we found overexpression of *TNF-α* in FOP PBMC in stabilized cell culture, which was further increased by AA. TNF-α may participate as an important inflammatory cytokine modulator of ossification during flare-up in FOP. Down regulation of *IL-10* did not suggest PBMC signaling involvement in FOP conditions. *In vivo* studies suggest that in the bone, the induction of osteoprotegerin levels and the suppression of RANKL mediated by TNF-α may represent an interrelated mechanism to prevent excessive loss of bone mass, assuming that TNF-α plays a role in the central regulation of bone mass in pathological conditions (*49*).

Inflammatory response to tissue damage is also regulated by the ANS through inflammatory reflex and signaling of the anti-inflammatory cholinergic pathway through vagus nerve, acetylcholine and CHRNA7 located in macrophages, dendritic cells, T and B lymphocytes, mast cells and basophils (*6,50,51*). In our study, ADBR2 blockade *in vitro* led to the downregulation of *CHRNA7* and increase of *TNF-α* , consistent with this path and consistent with earlier reports (*49*) looking like a paradoxical inflammatory regulation by TNF-α. Considering FOP PBMC in a prone inflammatory state (*47,52*), it is reasonable to assume TNF-α as a protagonist in the inflammatory process. However, reported benefits of AA+PP (FOPCON) for FOP patients (*10*), may suggest a balance of neuro-inflammatory equilibrium by ANS. Yet, control of *TNF-α* gene expression through CHRNA7 signaling of anti-inflammatory cholinergic pathway remains a question in the context of FOP.

For the first time, the main receptors of the RAS were investigated in FOP PBMC, due to the potential inflammatory and algesia involvement in various pathologies (*53*). *AGTR1*, *AGTR2*, *MAS* and *MRGD* genes were not shown to be FOP PBMC phenotypic markers, nevertheless, the interrelationship between β-adrenergic function and angiotensin axes is evident (*7*). PP downregulated *AGTR1* gene expression in FOP and control PBMC. Interestingly, AA+PP augmented expressions of *AGTR2* and *MAS* and downregulated *MRGD* and *AGTR1* genes in FOP PBMC, favoring the anti-inflammatory RAS axis. PP inhibits angiogenesis through downregulation of vascular endothelial growth factor (VEGF) expression in hemangioma-derived stem cells (*18*), thus it would do the same to HO, possibly by means of RAS regulation *via* beta-adrenergic antagonism. PP would lead to renin reduction and RAS axis, reducing vascular supply (*15*). It seems that increase of *AGTR2* and *MAS* and decrease of *AGTR1* genes in FOP PBMC affect inflammatory paths towards HO, reducing angiogenesis. Responses in FOP PBMC AA+PP may explain much of the mechanism of FOPCON in benefit FOP patients. However, the RAS cascade extends well beyond the two main counterbalancing axes. The angiotensin converting enzymes *ACE1* and *ACE2* genes might be directly involved in the process yet to be investigated in FOP (*7,15*). However, *MRGD* showed significant downregulation in PBMC FOP in response to all treatments. Alamandine peptide binds to *MRGD* receptor towards vasodilation (*7*), which might favor HO, but the hypothesis of MRGD participation in FOP may link mainly to its role in the algesia mechanism, which is not yet studied in FOP. This speculation is not verified yet, but *MRGD* downregulation in FOP PBMC by AA+PP may be linked to vasoconstriction and pain relief (*10*), must be considered in the rationale of future research. Modulation of RAS pathways should be considered in the control of inflammation, fibrogenesis and angiogenesis in FOP.

The main aspect of the present work is that FOP leukocyte phenotype is possibly modulated by AA and PP treatment. Interestingly, we present for the first time that, *ACTB* is expressed in FOP PBMC and FOP leads to an upregulation that is significantly sensitive to AA and PP treatment. β-actin, besides involvement with inflammation, must modulate structural aspects of the cellular framework, perhaps to promote diapedesis and control leukocyte migration, a function that will need to be better clarified (*54*). ACTB protein has been shown

to activate endothelial nitric oxide synthase (eNOS) to form Nitric oxide (NO), a pro-inflammatory signaling molecule, mediator in inflammation pathogenesis, that induces inflammation due to over production in abnormal situations. A clarification of the NO path hole is needed for FOP.

In conclusion, FOP is an intractable disease due to the ACVR1 mutation. The destination is an imbalance of interconnected complex molecular cascades with inflammatory consequences, culminating in outbreaks and abnormal bone formation. It becomes impossible to treat this disease just by targeting a pathway or blocking a receptor, because a complex imbalance of many genes, such as a poorly governed molecular seesaw, makes it difficult to balance or harmonize physiologically. Any attempt at a therapeutic target disrupts the rest of the molecular pathways. Achieving fine-tuning of the various key targets is necessary, but extremely difficult, making this disease so far devoid of effective treatment. A future attempt to treat FOP should consider a multi-target cocktail.

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