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

Deborah Ribeiro Nascimento¹, Suzana Lopes Bomfim Balaniuc¹, Durval Batista Palhares¹, Adam Underwood³, Marilene Garcia Palhares¹, Fabiana Alves³, Francisco Oliveira Vieira³, Elaine Maria Souza-Fagundes³, Liane De Rosso Giuliani¹, Paula Cristhina Niz Xavier¹, Helen Lima Del Puerto³, Robson Augusto Souza Santos³, Amy Milsted², Jose Mauro Brum⁵, Iandara Schettert Silva¹, Almir Sousa Martins¹,³,*

¹UFMS/ Faculty of Medicine, Campo Grande, MS, Brazil; ²Walsh University, Division of Mathematics and Sciences, North Canton, OH, USA; ³UFMG/ Department of Physiology and Biophysics, Belo Horizonte, MG, Brazil; ⁴Centro Universitário Metodista Izabela Hendrix- IMIH, Belo Horizonte, MG, Brazil; ⁵Procter & Gamble Health Care & Global Clinical Sciences, Mason, OH, USA.

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, ADCT9, AGTR2 and MAS, while downregulated ADRB2, RUNX2, ADCY1, SVCTI 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 ADBR1 and ADBR2 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 need to be clarified (8). 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).

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®, 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 X3 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 × 10⁶ or 1.5 × 10⁶ cells) cultured and stabilized for 24 hours in complete RPMI 1640 medium, maintained in a CO₂ incubator (5%) at 37°C (Thermo scientific / Forma Series II Water Jacket CO₂ 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 × 10^5 cells for Real-time PCR. Ascorbic acid (L (+) - Ascorbinsäure Zur Analyse; 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 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.

2.4. 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 (ssDNA) 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) |
|--------|------------------|---------------------------------|-------------|
| ACRV1  | activin A receptor type 1 | F<CTGCCCTCACTGGAGCTTCTCCATGCAAGG> R<CTGCTGCTCGGATGGGATACCC> | 100 |
| BMP4   | bone morphogenetic protein 4 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 89 |
| COL1   | collagen type I α1 chain | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 108 |
| COL3   | collagen type III α1 chain | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 77 |
| ADRA1B | α1 receptor β-1 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 80 |
| ADRA2B | α1 receptor β-2 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 78 |
| RUNX2  | RUNX family trans. factor 2 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 103 |
| TNFα   | tumor necrosis factor α | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 95 |
| ACTB   | β-actin mRNA | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 295 |
| ADCY1  | adenylyl cyclase 1 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 98 |
| ADCY2  | adenylyl cyclase 2 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 81 |
| ADCY7  | adenylyl cyclase 7 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 103 |
| ADCY9  | adenylyl cyclase 9 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 96 |
| SVCT1  | Sodium vit. C carrier type 1 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 90 |
| SVCT2  | Sodium vit. C carrier type 2 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 98 |
| AGTR1  | angiotensin II receptor type 1 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 98 |
| AGTR2  | angiotensin II receptor type 2 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 115 |
| MAS    | MAS1 proto-oncogene GPCR | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 115 |
| MRGD   | MAS related GPCR D | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 160 |
| CHRNA7 | Cholinergic a-7 subunit receptor | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 100 |
| IL10   | interleukin 10 | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 123 |
| ALPL   | alkaline phosphatase | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 86 |
| S26    | S26 ribosomal protein RNA | F<CTGAACTGAAAAGAAGAGAGGAGG> R<CTGCTGCTCGGATGGGATACCC> | 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). ssDNA 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 ssDNA 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^(-ΔΔCT) 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 ≤ 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 ACDY, 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 Cont. | FOP PP X Cont. | FOP PP X FOP | FOP PP X Cont. | FOP PPAA X Cont. | FOP PPAA X FOP | FOP PPAA X Cont. |
|-------|----------------|------------------|--------------|----------------|----------------|--------------|----------------|-------------------|----------------|------------------|
| ACVR1 | ↓ - ↑ ↑↑ ↑↑↑ | ↑ ↑ ↑ ↑ ↑ ↑ | ↓ - - - - | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| BMP4  | - - - - - | ↑ ↑ | - - - - | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |
| COL1  | ↑ ↑ ↑ ↑ ↑ | ↑ ↑ ↑ ↑ ↑ | ↑ ↑ ↑ ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| COL3  | ↓ ↓ - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| ADRB1 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| ADRB2 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| RUNX2 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| TGFα-a | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| ACTB  | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| ADCC2 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| ADCC7 | - - - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| ADCC9 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| SVCT1 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| SVCT2 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| AGTR1 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| AGTR2 | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ | ↑ ↑ |
| MAS   | - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| MRGD  | - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| CHRNA7 | - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| IL-10 | - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |
| ALPL  | - - | - - | - - | - - | - - | - - | - - | - - | - - | - - |

Modulation (arrows); (-) not significant; ↑↓ = up/downregulation (p ≤ 0.05); ↑↑↓↓ = up/downregulation (p = 0.001 to ≤ 0.01); ↑↑↑↓↓↓ = up/downregulation (p < 0.001); (N) not experimented.

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 *ADRB1* and *ADRB2* (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 *ADRB2*, 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 trauma which trigger HO. Cellular models, including Epstein-Barr transformed lymphoblast cell lines, dental pulp
Intractable & Rare Diseases Research
Advance Publication

Intractable & Rare Diseases Research
Advance Publication

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 adrenergic 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 neuropetide 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 pro-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 skeletalgenesis, 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 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 vasocostriction 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.

Acknowledgements

This study is part of Nascimento DR Ph.D. thesis and Balaniuc SLB Msc dissertation at Graduate Program in Health and Development in the Central West Region of MS, UFMS/Brazil-PPGSD. We thank the patients and volunteers who contributed to this research.

Funding: This work was supported by grant 2016-009 from Instituto de Assistência em Pesquisa, Educação e Saúde - IAPES, Campo Grande, MS, Brazil.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Kaplan FS, Pignolo RJ, Al Mukaddam MM, Shore EM. Hard targets for a second skeleton: therapeutic horizons for fibrodysplasia ossificans progressiva (FOP). Expert Opin Orphan Drugs. 2017; 5:291-294.
2. Shore EM, Xu M, Feldman GJ, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet. 2006; 38:525-527.
3. Hino K, Horigome K, Nishio M, Komura S, Nagata S, Zhao C, Jin Y, Kawakami K, Yamada Y, Ohta A, Tochida J, Ikeya M. Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva. J Clin Invest. 2017; 127:3339-3352.
4. Hildebrandt L, Gaber T, Kühen P, Morhart R, Unterbörsch H, Schomburg L, Seemann P. Trace element and cytokine concentrations in patients with Fibrodysplasia Ossificans Progressiva (FOP): A case control study. J Trace Elem Med Biol. 2017; 39:186-192.
5. Salisbury E, Sonnet C, Heggeness M, Davis AR, Olmsted-Davis E. Heterotopic ossification has some nerve. Crit Rev Eukaryot Gene Expr. 2010; 20:313-324.
6. Rosas-Ballina M, Tracey KJ. Cholinergic control of inflammation. J Intern Med. 2009; 265:663-679.
7. Santos RAS, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS axis of the renin-angiotensin system: focus on Angiotensin-(1-7). Physiol Rev. 2018; 98:505-553.
8. Wentworth KL, Masharani U, Hsiao EC. Therapeutic advances for blocking heterotopic ossification in fibrodysplasia ossificans progressiva. Br J Clin Pharmacol. 2019; 85:1180-1187.
9. Haupt J, Xu M, Shore EM. Variable signaling activity by FOP ACVR1 mutations. Bone. 2018; 109:232-240.
10. Palhares DB, Nascimento DR, Palhares MG, Balaniuc SLB, Giuliani LR, Xavier PCN, Brum JMG, Alves F, Vieira FO, Souza-Fagundes EM, Underwood A, Milsted A, Santos RAS, Martins AS. Propranolol and ascorbic acid in control of fibrodysplasia ossificans progressiva flare-ups due to accidental falls. Intractable Rare Dis Res. 2019; 8:24-28.
11. Arrigoni O, De Tullio MC. Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta. 2002; 1569:1-9.
12. Ashino H, Shimamura M, Nakajima H, Dombou M, Kawanaka S, Oikawa T, Iwaguchi T, Kawashima S. Novel function of ascorbic acid as an angiotostatic factor. Angiogenesis. 2003; 6:259-269.
13. Belin S, Kaya F, Burtey S, Fontes M. Ascorbic acid and gene expression: another example of regulation of gene expression by small molecules? Curr Genomics. 2010; 11:52-57.
14. Fernandes G, Barone AW, Dzial R. The effect of ascorbic acid on bone cancer cells in vitro. Cogent Biol. 2017; 3:1288335.
15. Stiles JM, Amaya C, Rains S, Diaz D, Pham R, Battiste J, Modiano JF, Kotka V, Boucheron LE, Mitchell DC, Bryan BA. Targeting of beta adrenergic receptors results in therapeutic efficacy against models of hemangioendothelioma and angiosarcoma. PLoS One. 2013; 8:e60021.
16. Nascimento DR, Giuliani LR, Palhares MG, Alves F, Martins SF, Vieira FO, Brum JMG, Giuliani LR, Palhares MG, Underwood A, Souza-Fagundes EM, Milsted A, Santos RAS, Martins AS. Ascorbic acid modulates the expression of genes involved in heterotopic ossification. NBC-Periódico Científico do Núcleo Biociências. 2017; 7:81-97.
17. Hajighasemi F, Mirshafiey A. Propranolol effect on proliferation and vascular endothelial growth factor secretion in human immunocompetent cells. J Clin Immunol Immunopathol Res. 2010; 2:22-27.
18. Zhang L, Mai HM, Zheng J, Zheng JW, Wang VA, Qin ZP, Li KL. Propranolol inhibits angiogenesis via down-regulating the expression of vascular endothelial growth factor in hemangioma derived stem cell. Int J Clin Exp Pathol. 2013; 7:48-55.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2\(^{-\Delta\Delta CT}\) method. Methods. 2001; 25:402-408.
20. Billings PC, Fiori JL, Bentwood JL, O’Connell MP, Xiao N, Nussbaum B, Caron RJ, Shore EM, Kaplan FS. Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from...
patients with fibrodysplasia ossificans progressiva (FOP). J Bone Miner Res. 2008; 23:305-313.

21. Micha D, Voermans E, Eekhoff MEW, van Essen HW, Zandieh-Doublabi B, Netelenbos C, Rustemeeyer T, Sistermans EA, Pals G, Bravenboer N. Inhibition of TGFβ signaling decreases osteogenic differentiation of fibrodysplasia ossificans progressiva fibroblasts in a novel in vivo model of the disease. Bone. 2016; 84:169-180.

22. Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, Levine M. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci. 2005; 102:13604-9.

23. Padayatty SJ, Levine M. Vitamin C physiology: the know and the unknown and Goldilocks. Oral Dis. 2016; 22:463-493.

24. Gonzalez JP, Calvo R, Rodriguez-Sasiaín JM, Jimenez R, Aguirre C, Valdivieso A, du Souch P. Influence of vitamin C on the absorption and first pass metabolism of propranolol. Eur J Clin Pharmacol. 1995; 48:295-297.

25. Eslami M, Badkoubet HS, Mousavi M, Radmehr H, Salehi M, Tavakoli N, Avadi MR. Oral ascorbic acid in combination with beta-blockers is more effective than beta-blockers alone in the prevention of atrial fibrillation after coronary artery bypass grafting. Tex Heart Inst J. 2007; 34:268-274.

26. Ludwig M-G, Seuwen K. Characterization of the human adenylyl cyclase gene family: cDNA, gene structure, and tissue distribution of the nine isoforms. J Recept Signal Transduct Res. 2002; 22:79-110.

27. Passage E, Norreel JC, Noack-Fraissignes P, Sanguedolce F, Baerwald CGO. Beta 2-adrenergic receptors mediate expressed form of adenylyl cyclase. J Biol Chem. 1996; 271:13900-13907.

28. Hanoune J. Identification and characterization of a widely tissue distribution of the nine isoforms. J Recept Signal Transduct Res. 2002; 22:79-110.

29. Premont RT, Matsuoka I, Mattei MG, Pouille Y, Defer N, Breton E. BMP signaling mediated by activating pathway of adenylyl cyclase stimulation. Shock. 2003; 19:108-112.

30. Wåhle M, Neumann RP, Moritz F, Krause A, Buttgereit F, Baerwald CGO. Beta 2-adrenergic receptors mediate the differential effects of catecholamines on cytokine production of PBMC. J Interferon Cytokine Res. 2005; 25:384-394.

31. Bernardin G, Kísok Sa, Delporte C, Robberecht P, Vincent J-L. Impairment of beta-adrenergic signaling in healthy peripheral blood mononuclear cells exposed to serum from patients with septic shock: involvement of the inhibitory pathway of adenylyl cyclase stimulation. Shock. 2003; 19:108-112.

32. Togari A. Adrenergic regulation of bone metabolism: possible involvement of sympathetic innervation of osteoblastic and osteoclastic cells. Microsc Res Tech. 2002; 58:77-84.

33. Pierroz DD, Bonnet N, Bianchi EN, Bouxsein RL, Baldock PA, Rizzoli R, Ferrari SL. Deletion of β-adrenergic receptor 1, 2, or both leads to different bone phenotypes and response to mechanical stimulation. J Bone Miner Res. 2012; 27:1252-1262.

34. Scanzano A, Cosentino M. Adrenergic regulation of innate immunity: a review. Front Pharmacol. 2015; 6:171.
stimulus: A pilot study in healthy subjects. Genes Nutr. 2014; 9:390.

49. Mito K, Sato Y, Kobayashi T, Miyamoto K, Nitta E, Iwama A, Matsumoto M, Nakamura M, Sato K, Miyamoto T. The nicotinic acetylcholine receptor α7 subunit is an essential negative regulator of bone mass. Sci Rep. 2017; 7:45597.

50. Zdanowski R, Krzyzowska M, Ujazdowska D, Lewicka A, Lewicki S. Role of α7 nicotinic receptor in the immune system and intracellular signaling pathways. Cent Eur J Immunol. 2015; 40:373-379.

51. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. Immunol Rev. 2012; 248:188-204.

52. Del Zotto G, Antonini F, Azzari I, Ortolani C, Tripodi G, Giacopelli F, Cappato S, Moretta L, Ravazzolo R, Bocciardi R. Peripheral blood mononuclear cell immunophenotyping in fibrodysplasia ossificans progressiva patients: evidence for monocyte DNAM1 upregulation. Cytometry B Clin Cytom. 2018; 94:613-622.

53. Lan L, Xu M, Li J, Liu L, Xu M, Zhou C, Shen L, Tang Z, Wan F. Mas-related G protein-coupled receptor D participates in inflammatory pain by promoting NF-κB activation through interaction with TAK1 and IKK complex. Cell Signal. 2020; 76:109813.

54. Kondrikov D, Fonseca FV, Elms S, Fulton D, Black SM, Block ER, Su Y. β-actin association with endothelial nitric-oxide synthase modulates nitric oxide and superoxide generation from the enzyme. J Biol Chem. 2010; 285:4319-4327.

Received January 15, 2021; Revised April 2, 2021; Accepted April 29, 2021.

*Address correspondence to:*

Almir Sousa Martins, UFMG/ Department of Physiology and Biophysics, Av Antonio Carlos, 6627, A4-256, Belo Horizonte, MG, Brasil - 31.270-900.

E-mail: alisbetermster@gmail.com; asm2011@ufmg.br

Released online in J-STAGE as advance publication May 22, 2021.