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Gene modulation in buccal epithelial cells of patients with fibrodysplasia ossificans progressiva treated with ascorbic acid and propranolol

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Abstract

Introduction and Objective. Fibrodysplasia Ossificans Progressiva (FOP) is an ultra-rare genetic disease which at birth may present hallux brevis and valgus as pathognomonic signs. Post-natal heterotopic ossification (HO) progresses into disabling, inflammatory flare-ups. Mutated ACVR1 gene (e.g.: c.617G>A) dysregulates the bone morphogenic protein (BMP) signaling pathway, alters SMAD and p38 MAPK phosphorylation downstream cascades, while Activin-A, through ACVR1 (R206H), signals BMP through Smad1/5 and the TGF-β pathways, activating Smad2/3 canonical cascades, converging to a fate of RUNX2 activation into HO. Expansion of therapeutic possibilities aim to other inflammation pathways. The renin-angiotensin system (RAS) is a putative candidate. Its inflammatory axis may converge along FOP canonic ones. Yet, FOP biological specimen collection for molecular studies is an obstacle due to the risk of flare-up. The aim of the study is to evaluate RAS receptors, as well as potential inflammatory and osteogenic target genes.

Materials and Method. Buccal epithelial cell scrapings (BEC) were collected from healthy controls, untreated FOP patients and 90 days AA+PP treated patients. BEC total RNA was obtained for RT-qPCR gene expression analysis.

Results. Increased expressions in basal *TNFα* and *ADRβ2*, plus tendency of *RUNX2*, genes in BEC of untreated FOP patients compared with healthy control volunteers were confirmed. *AT1R*, *AT2R*, *MAS1* and *MRGD* genes appeared undifferentiated in BEC FOP at baseline, while AA+PP downregulated these four RAS receptor (RASr) genes to levels below controls and normalized *TNFα*, *ADRβ2* and *RUNX2*.

Conclusions. The results imply that AA+PP can modulate genes in FOP BEC non-canonical physiopathology pathways related to inflammation, cell signaling and osteogenesis processes.

Key words

heterotopic ossification, ascorbic acid, propranolol, fibrodysplasia ossificans progressiva, mouth epithelial cells, FOP inflammation

INTRODUCTION

Fibrodysplasia Ossificans Progressiva (FOP), OMIM #135100, is an ultra-rare, untreatable, autosomal dominant genetic disease with classic presentation marked by hallux brevis and/or hallux valgus presence at birth. FOP progressive symptoms develop as a result of recurrent, spontaneous, or provoked inflammatory flare-ups, leading to tragic and disabling post-natal heterotopic ossification (HO). Intercurrent painful nodules, which follow an anatomical and chronological pattern of appearance and progression, arise in the soft tissues adjacent to the skeletal tissue, progressing in an intense and disordered inflammatory process towards chondrogenesis, and subsequent replacement of the soft tissue by true bone endochondral HO [1,2]. Mutations in the ACVR1 gene (e.g.: c.617G>A, p.R206H) may cause dysregulation of

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the bone morphogenic protein (BMP) signaling pathway, decreasing BMP antagonist expression, and increasing specific proteins SMAD and p38 MAPK phosphorylation. There is an activation of the BMP pathway in the absence of ligand, and ligand-dependent hyperactivity. Furthermore, Activin-A has been recognized as an agonist of the BMP pathway, in the presence of ACVR1(R206H), signaling the BMP pathway through activation of Smad1/5 and the TGF-β pathway through activation of Smad2/3, and with a likely role in the continued expansion of HO in FOP (2). However, the fate of all these canonical cascades effectively culminates in RUNX2-dependent pro-osteoblast differentiation, which involves modulation of TNFα in macrophages via stimulation of β-adrenergic receptors, in local signaling pathways for neurogenesis, angiogenesis, in an inflammatory complex, with an anomalous osteogenic end [3].

FOP within its clinical implications is an inflammatory disease. Corticosteroids, cyclooxygenase 2 (COX-2) inhibitors, and non-steroidal anti-inflammatory drugs are restricted to acute symptomatic treatment, especially of flare-ups, with negligible effect on FOP chronic pain and progression of ectopic ossification. Interruption and minimization of FOP natural progression are immediate goals. The most advanced therapy in clinical trials is Palovarotene, a RARγ agonist. Associated with mild mucocutaneous adverse events, it has been approved for use in three countries, restricted to patients aged eight to ten, given the impairment in skeletal growth detected in other phases of the trial [2,4]. Still with great expectation, anti-activin A antibodies (REGN2477; Garetosmab) and mTOR pathway inhibitors (Rapamycin) have shown equally promising results, completing phases III and II of clinical trials, respectively. Garetosmab, so far tested only in adults, has been associated with mild recurrent epistaxis, madarosis and skin infections [5]. Rapamycin inhibits chondrogenesis, but continued use is associated with significant adverse effects, which are still being investigated in FOP [4,6]. Understanding HO as a sum of multi-factorial molecular disorders is important to expand therapeutic possibilities by investigating other signaling pathways involved in inflammation and pain.

By in vitro experimental approach, peripheral blood mononuclear cell (PBMC) culture model was checked to evaluate putative FOP gene targets within canonic and non-canonic physiopathology pathways. Various gene transcripts highlighted other non-canonical pathways of FOP pathophysiology through qPCR, suggesting that FOP involves complex, interlinked inflammatory cascades that could hinder therapeutic approaches. In this case, the canonic pathways therapeutic target would unbalance other specific molecular pathways, resulting in possible collateral clinical consequences. A FOP characteristic gene expression profile was observed, and it was hypothesized that the use of ascorbic acid (AA) plus propranolol (PP) in FOP could be beneficial for modulating the expression of regulatory genes of inflammation, angiogenesis and osteogenesis, including beta-adrenergic receptors genes and components of the renin-angiotensin system (RAS) [7]. However, genetic modulation assessments for real-time monitoring of experimental therapies are not feasible due to the difficulty of obtaining biological material from FOP patients without exposing them to the risk of flare-ups, inherent in sample collection by invasive methods. In this regard, the use of buccal epithelial cells (BEC) was recently proposed by the authors as a non-invasive and viable specimen for gene expression studies [8].

The aim of the study is to evaluate the modulation of *TNFa*, *ADRB2*, *RUNX2* and RAS receptor genes (*AGTR1*, *AGTR2*, *MAS1* and *MRGD*) in buccal epithelial cells of FOP patients treated with AA and PP.

MATERIALS AND METHOD

Population, treatment and samples. Partial data from a parallel clinical trial (Rebec RBR-9dkn9ry)were used, consisting of healthy individuals in the control group (n=13); untreated patients with FOP (n=7); and patients with FOP undergoing treatment for symptom control (n=8) (FOPCON – Patent BR1020150324928). The study was approved by the Research Ethics Committee of the Federal University of Mato Grosso do Sul (UFMS) (CAEE 40606820.5.0000.0021), and participants were enrolled after clarification of and signing an informed consent form (TCLE/TALE). BEC samples were collected by scraping the inner side of each cheek, using a sterile paediatric tongue depressor, as described previously [8]. BEC was immediately eluted in 500 µl of RNAlater® (Ambion™), transported at room temperature and stored in a freezer at -20 °C until processing. Samples from FOP volunteers treated with the AA+PP compound (FOPCON) were collected in 2 stages: 1] 90 days of AA+PP treatment and 2] 5 days medication interruption (MI). Individuals with FOP received individualized doses of AA+PP for 3 months, prescribed according to weight and age (9). For participants under 18 years of age, the dose of propranolol did not exceed 2 mg/kg/day. For adults, the dose of propranolol ranged from 14 – 17 mg per capsule, ingested 3 times a day. The concentration of ascorbic acid did not exceed the dose of 250 mg per capsule, also ingested 3 times a day.

Total RNA extraction and DNase treatment. Briefly, thawed samples were centrifuged at 700g, 4 °C for 15 minutes, and pelleted cells were RNA extracted by Trizol® method, resuspended in 80 µl of DEPC RNAse-free water. Total RNA concentration was determined from 2 µl aliquots in a 260 nm NanoDropTMOne spectrophotometer (Thermo ScientificTM Spectrophotometer) quantification. RNA samples were treated with DNAse I (TURBO DNA-free kit, Ambion Inc., Foster, California, USA), according to manufacturer's protocols. DNAse-treated RNA was resuspended in 25 µl of DEPC H2O aliquots and quantified at 260 nm for later use in RT-PCR.

Oligonucleotide primers. Target genes *AGTR1*, *AGTR2*, *MAS1* and *MRGD*; *TNF-α*, *RUNX2* and *ADRB2* were selected for relative gene expression study. Reverse transcription (RT) and quantitative real-time PCR (qPCR) oligonucleotide primers are shown in Table 1. Primers were designed and selected by the authors from available mRNA sequences from GenBank using the BLASTn programme (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and synthesized by IDT (Integrated DNA Technologies). Synthetized primers were received lyophilized, then resuspended in sterile filtered nuclease-free water (0.22 µm; q.s. 100 pmol/µl) and stored in aliquots of 10 pmol/µl at -20 °C. The S26 gene was used as qPCR endogenous normalizer.

Reverse transcription and Qpcr. RT for first-stranded complementary DNA (cDNA) synthesis was performed from ~350 ng of total RNA; preincubated at 70 °C for 10 min with 10 pmol of each reverse primer (RNA/primers) plus 10 pmol of oligo dT20 primer (Invitrogen), followed by storage on ice

Table 1. Targeted genes and selected oligonucleotide primers

Gene	mRNA Description	Oligonucleotide primer sequences (<5´to >3´)	Target (bp)
AGTR1	angiotensin II receptor type 1	F<TTCAGCCAGCGTCAGTTTCA>R<GGCGGGACTTCATTGGGT>	101
AGTR2	angiotensin II receptor type 2	F<TATGGCCTGTTTGTCCTCATTG>R<CCATTGGGCATATTTCTCAGGT>	115
MAS1	MAS1 proto-oncogene receptor	F<GCTACAACACGGGCCTCTATCTG>R<TACTCCATGGTGGTCACCAAGC>	160
MRGD	MAS related GPCR family member D	F<TCCCTGCCTCTGAGCATCTA>R<GAGAGGCGTGACAAGCTGAA>	100
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<GTTTGCTACAACATGGGCTAC	95
S26	S26 ribosomal protein RNA	F<TGTGCTTCCCAAGCTGTATGTGAA>R<CGATTCCTGACTACTTTGCTGTG	75

(**Bp**) base pairs ; **(F)** forward (sense); **(R)** reverse (anti-sense)

on the benchtop. 10.4 µl of reverse transcriptase enzyme mix (40 U) in RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) containing 4 µl of dNTP mix (10 mM of each) were incubated at 45 °C for 1 h with RNA/primers previously described. The reaction stopped at 4 °C and the cDNA stored at -20° until qPCR. All reagents were from Invitrogen™ (SuperScript™ First-Strand Synthesis System for RT-qPCR). RT cDNA samples were used in qPCR, in a QuantStudioTM 6 Flex Real-Time PCR System, using the reaction protocol described by the SYBR Green PCR Master Mix Kit (THERMO FISHER SCIENTIFIC, 2022). Duplicates of 3 µl BEC cDNA 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 by adding 7 µl of Sybr Mix (5 µl of SYBR Green PCR Master Mix Kit; 2 µl of each primer [sense and antisense] at 0.3 pmol/µl). The plate was sealed with optical adhesive (ABI PRISM® Optical Adhesive Covers, Invitrogen Life Technologies, Carlsbad, CA, USA). qPCR was performed in the standard thermal cycling protocol: [stage 1] one cycle of 50 °C/2 min; [stage 2] one cycle at 95 °C/10 min; [stage 3] 40 cycles of 95 °C/15 s, followed by a dissociation curve.

Gene relative quantification and statistical analysis. The gene expression relative quantification was determined by comparative analysis with the endogenous control, using the comparative CT method, as well as the 2-ΔΔCT method for relative gene expression levels, as described elsewhere (7). The data were analyzed using the GraphPad Prism 5 statistical program for T-student and 1-way ANOVA. The results were statistically significant when $p < 0.05$.

RESULTS

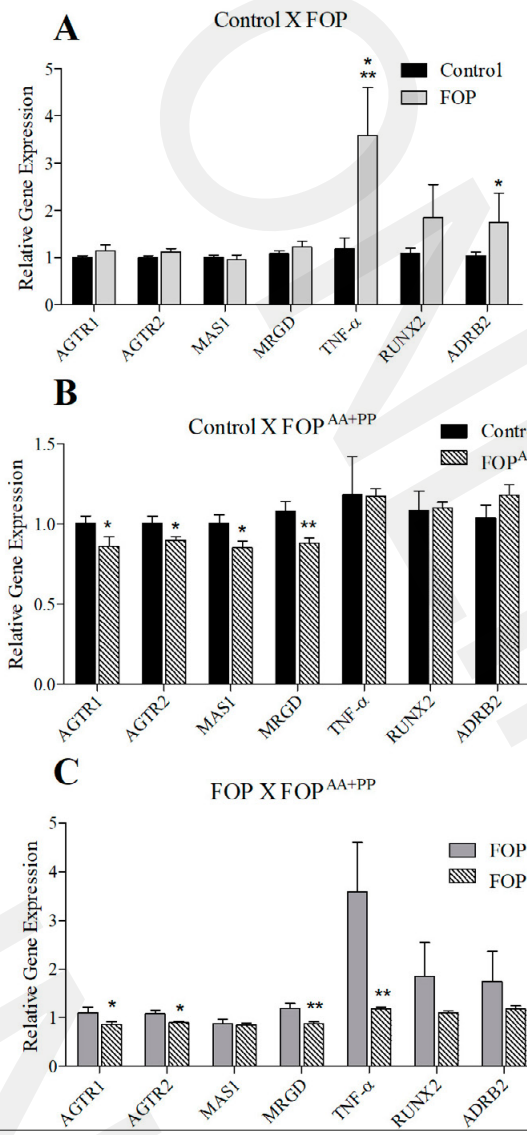
Population and haemodynamic state. The ages of untreated FOP, treated FOP, and healthy control groups ranged from 4–62, 4–39, and 7–68 years, respectively, with no significant differences in median of ages between the groups (p=0.658; Kruskal-Wallis). Continuous use of AA+PP for 90 days caused slight but significant changes in the vital haemodynamic states of FOP patients, as observed by random daily monitoring, i.e., at any time of the day, by measuring the blood pressure and heart rates of the patients. The average heart rate values (bpm) measured during the 3 stages of treatment were 100 ± 4.36 at month 1, 89 ± 2.95 at month 2, and 90 ± 3.68 at month 3. Mean values of mean arterial pressure (mmHg) at months 1, 2 and 3 were: 75 ± 4.33; 72 ± 4.19 and 80 ± 3.19, respectively.

Basal expressions and effects of AA+PP treatment. Results of comparisons of basal expressions of the target genes evaluated in BEC of FOP patients, without any medical treatment, compared with BEC of healthy control volunteers are presented in Figure 1A. The relative expression profiles of the genes *AGTR1*, *AGTR2*, *MAS1* and *MRGD* showed no significant differences when compared to the control group. However, significantly increased expressions of the genes *TNF-α* (p=0.0006) and *ADRB2* (p=0.0424) and a trend for *RUNX2* (p=0.071) were observed in the FOP group, compared to the expression profiles of the control group. Figures 1B and 1C show the results of the expression profiles of the seven genes evaluated, showing the effect of treating FOP patients with AA+PP, compared with the basal expressions of healthy control and untreated FOP, respectively. The results of AA+PP treatment for 90 days showed down-regulation of the genes *AGTR1* (p=0.041), *AGTR2* (p=0.028), *MAS1* (p=0.018) and *MRGD* (p=0.007) in relation to the respective genes in BEC of healthy controls, and of *AGTR1* (p=0.044), *AGTR2* (p=0.012), *MRGD* (p=0.009) and *TNF-α* (p=0.005) in relation to the untreated FOP group.

Effect of medication interruption. Figures 2A, 2B and 2C show the effect of medication interruption (MI) for 5 consecutive days on the expression of the 7 genes evaluated, compared to the basal expressions of the healthy control, untreated FOP and treated FOP groups, respectively. Medication interruption for 5 days resulted in an expression profile similar to that of the treated FOP group, with significant differences in the down-regulation of *AGTR1* (p=0.0095), *AGTR2* (p=0.002), *MAS1* (p=0.034), *MRGD* (p=0.004), and in the immediate return to the over-expression status of *ADRB2* (p=0.0008), when compared to the basal expression of the healthy control group. Significant down-regulations were still maintained for the genes *AGTR1* (p=0.016), *AGTR2* (p=0.002), *MRGD* (p=0.009) and *TNF-α* (p=0.030), even with 5 days of interruption of AA+PP, when compared to the untreated FOP group.

DISCUSSION

The study monitored FOP patients over 90 days of continuous AA+PP use at individually recommended doses, showing stable heart rates and blood pressure. Previous observations inferred lower recurrence of flare-ups, clinical improvement of pain symptoms, mental and physical disposition, better quality of sleep and absence of side-effects [9]. AA+PP effect on the expression of genes involved in inflammation and



Group	Control	FOP		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	1.005 ± 0.045 N=6	1.102 ± 0.119 N=5	0.2171	-
AGTR2	1.005 ± 0.044 N=6	1.086 ± 0.070 N=5	0.1686	-
MAS1	1.007 ± 0.050 N=6	0.882 ± 0.090 N=5	0.1189	-
MRGD	1.082 ± 0.062 N=5	1.186 ± 0.109 N=5	0.2147	-
TNF-α	0.980 ± 0.125 N=12	3.588 ± 1.015 N=5	0.0006	↑
RUNX2	1.086 ± 0.121 N=13	1.847 ± 0.703 N=6	0.0715	↑
ADRB2	1.038 ± 0.080 N=13	1.748 ± 0.618 N=5	0.0424	↑

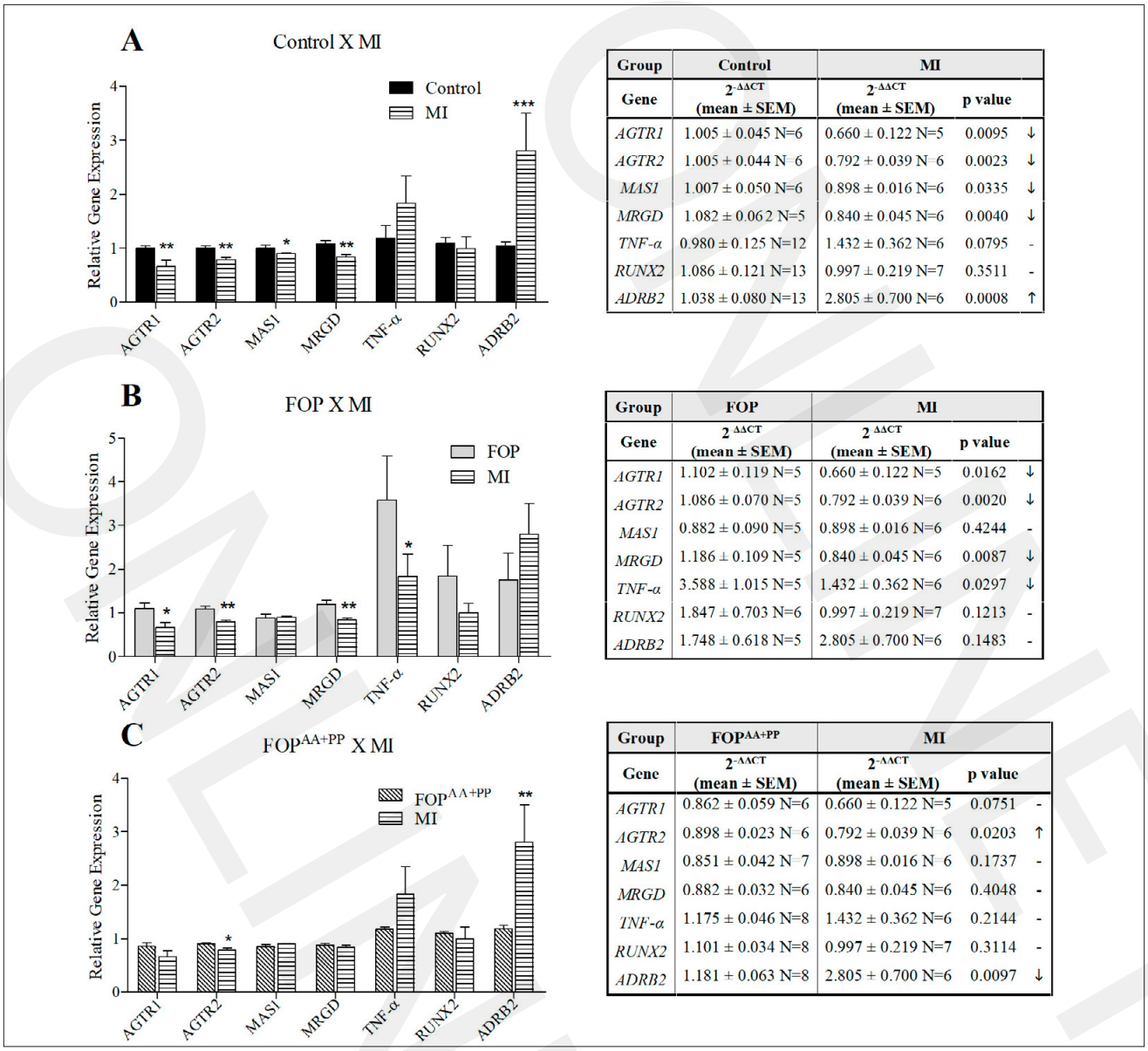
Group	Control	FOP^{AA+PP}		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	1.005 ± 0.045 N=6	0.862 ± 0.059 N=6	0.0406	↓
AGTR2	1.005 ± 0.044 N=6	0.898 ± 0.023 N=6	0.0284	↓
MAS1	1.007 ± 0.050 N=6	0.851 ± 0.042 N=7	0.0179	↓
MRGD	1.082 ± 0.062 N=5	0.882 ± 0.032 N=6	0.0070	↓
TNF-α	0.980 ± 0.125 N=12	1.175 ± 0.046 N=8	0.1178	-
RUNX2	1.086 ± 0.121 N=13	1.101 ± 0.034 N=8	0.4625	-
ADRB2	1.038 ± 0.080 N=13	1.181 ± 0.063 N=8	0.1123	-

Group	FOP	FOP^{AA+PP}		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	1.102 ± 0.119 N=5	0.862 ± 0.059 N=6	0.0444	↓
AGTR2	1.086 ± 0.070 N=5	0.898 ± 0.023 N=6	0.0115	↓
MAS1	0.882 ± 0.090 N=5	0.851 ± 0.042 N=7	0.3706	-
MRGD	1.186 ± 0.109 N=5	0.882 ± 0.032 N=6	0.0086	↓
TNF-α	3.588 ± 1.015 N=5	1.175 ± 0.046 N=8	0.0052	↓
RUNX2	1.847 ± 0.703 N=6	1.101 ± 0.034 N=8	0.1194	-
ADRB2	1.748 ± 0.618 N=5	1.181 ± 0.063 N=8	0.1320	-

Figure 1. Basal expressions and AA+PP treatment gene modulation effects. (A) Basal target genes expressions in BEC of untreated FOP patients compared to BEC of healthy control volunteers. RAS receptor genes AGTR1, AGTR2, MAS1 and MRGD show equal expressions as control group. Increased TNF-α (p=0.0006) and ADRB2 (p=0.0424) and a trend for RUNX2 (p=0.071, red arrow) expressions are noted in FOP group. Expression profiles after AA+PP treatment of FOP patients are compared to basal expressions of healthy control (B) and to untreated FOP (C), respectively. AA+PP treatment for 90 days showed downregulation of the genes AGTR1 (p=0.041), AGTR2 (p=0.028), MAS1 (p=0.018) and MRGD (p=0.007) in relation to the respective genes in BEC of healthy controls, and of AGTR1 (p=0.044), AGTR2 (p=0.012), MRGD (p=0.009) and TNF-α (p=0.005) in relation to the untreated FOP. Side tables are complementary to respective comparisons

heterotopic ossification of FOP were evaluated by means of qPCR from BEC total RNA. BEC results corroborated data from previous studies in both PBMC [7] and in BEC [8], regarding the prevalence of a basal gene expression profiles characteristic of FOP patients. However, as a novelty, a modulation of BEC FOP target genes occurred with AA+PP treatment. Interestingly, the compound AA+PP, in addition to down-regulating *TNF-α*, *ADRB2* and normalizing *RUNX2*, caused down-regulation of the genes encoding the main RAS receptors AGTR1, AGTR2, MAS1 and MRGD. TNF-α is a cytokine expressed primarily by activated macrophages, an immune messenger that, when excessive, plays critical roles in several inflammatory diseases. It is also produced in several other cell types, such as T cells, B cells, dendritic cells, and mast cells. It is rapidly produced in response to pathogens, cytokines, and environmental stressors [10]. Due to its ove-expression in previous studies by the authors in PBMC in vitro and in BEC in vivo, they consider it a possible target gene for studies aimed at understanding and treating the clinical pathophysiology of FOP [7,8].

Both AA and PP have been widely reported separately to modulate different genes in various pathological conditions. The probable actions and rationality of using the AA+PP composition in minimizing FOP symptoms have been presented and discussed in previous works [9]. AA, for instance, has been shown to be active in all stages of wound healing processes, especially in the first inflammatory stage, mainly down-regulating several pro-inflammatory genes, including *TNF-α* [11]. On the other hand, β2-receptors signaling pathway play a crucial role in the production of pro-inflammatory cytokines, macrophage activation, and B-cells, to the production of antibodies with inflammation exacerbation. Therefore, β-blockers, such as PP, have anti-inflammatory effects through reduction of pro-inflammatory cytokines release through inhibition of NF-kB target pathways [12].



Group	Control	MI		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	1.005 ± 0.045 N=6	0.660 ± 0.122 N=5	0.0095	↓
AGTR2	1.005 ± 0.044 N=6	0.792 ± 0.039 N=6	0.0023	↓
MAS1	1.007 ± 0.050 N=6	0.898 ± 0.016 N=6	0.0335	↓
MRGD	1.082 ± 0.062 N=5	0.840 ± 0.045 N=6	0.0040	↓
TNF-α	0.980 ± 0.125 N=12	1.432 ± 0.362 N=6	0.0795	-
RUNX2	1.086 ± 0.121 N=13	0.997 ± 0.219 N=7	0.3511	-
ADRB2	1.038 ± 0.080 N=13	2.805 ± 0.700 N=6	0.0008	↑

Group	FOP	MI		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	1.102 ± 0.119 N=5	0.660 ± 0.122 N=5	0.0162	↓
AGTR2	1.086 ± 0.070 N=5	0.792 ± 0.039 N=6	0.0020	↓
MAS1	0.882 ± 0.090 N=5	0.898 ± 0.016 N=6	0.4244	-
MRGD	1.186 ± 0.109 N=5	0.840 ± 0.045 N=6	0.0087	↓
TNF-α	3.588 ± 1.015 N=5	1.432 ± 0.362 N=6	0.0297	↓
RUNX2	1.847 ± 0.703 N=6	0.997 ± 0.219 N=7	0.1213	-
ADRB2	1.748 ± 0.618 N=5	2.805 ± 0.700 N=6	0.1483	-

Group	FOP^{AA+PP}	MI		
Gene	$2^{-\Delta\Delta CT}$ (mean ± SEM)	$2^{-\Delta\Delta CT}$ (mean ± SEM)	p value	
AGTR1	0.862 ± 0.059 N=6	0.660 ± 0.122 N=5	0.0751	-
AGTR2	0.898 ± 0.023 N=6	0.792 ± 0.039 N=6	0.0203	↑
MAS1	0.851 ± 0.042 N=7	0.898 ± 0.016 N=6	0.1737	-
MRGD	0.882 ± 0.032 N=6	0.840 ± 0.045 N=6	0.4048	-
TNF-α	1.175 ± 0.046 N=8	1.432 ± 0.362 N=6	0.2144	-
RUNX2	1.101 ± 0.034 N=8	0.997 ± 0.219 N=7	0.3114	-
ADRB2	1.181 + 0.063 N=8	2.805 + 0.700 N=6	0.0097	↓

Figure 2. Modulation effects of AA+PP five days treatment interruption (MI). Target genes modulation effects after MI are demonstrated by comparisons between healthy control vs MI (A), untreated FOP vs MI (B) and treated FOP vs MI (C), respectively. Side tables are complementary to respective comparisons. AA+PP interruption resulted in immediate return to the overexpression status of ADRB2 (p=0.0008)

Furthermore, AA is a redox regulator in several physiological processes. In addition to being an antioxidant, it modulates the expression of immunoregulatory genes, acting as a co-factor for histone demethylases, dependent on α-ketoglutarate containing Fe+2, as well as in the epigenetic modulation mediated by methylcytosine dioxygenase. In these mechanisms, AA facilitates the differentiation of double-negative T cells into double-positive T cells and of helper T cells in different pathways, modulating the differentiation of plasma cells and promoting the early differentiation of haematopoietic stem cells into NK cells, thus participating in the regulation of innate and adaptive immune cells, and can be used as an adjuvant therapy in several disorders of the immune and inflammatory systems [13]. However, there is still a long way to go in understanding the cellular immune and immunoinflammatory mechanisms of FOP pathophysiology. Probably, in vitro and in vivo studies using AA, which is non-toxic, can help us to better understand the participation of the immune system in the immuno-inflammatory pathways of FOP. The fact is that in the authors' previous PBMC in vitro [7] and current in vivo BEC experiments, both pro-inflammatory genes *TNFα* and *ADRB2* were categorically down-regulated by AA and PP alone or together in the former, as well as their combination AA+PP, in the latter.

No less important are the current studies demonstrating the crosstalk of the RAS with several canonical and non-canonical inflammatory cell signaling pathways. The role of RAS in FOP has not yet been described or clearly evaluated, as well as its anti-inflammatory potential as a therapeutic target. The present work is the beginning of this challenge. Interestingly, the basal relative expression profiles of the *AGTR1*, *AGTR2*, *MAS1* and *MRGD* genes, in untreated FOP showed no significant differences compared to healthy controls. However, after 90 days of AA+PP treatment, genes were down-regulated followed by a medication interruption

for only 5 days, which did not alter the down-regulation effect of the *AGTR1*, *AGTR2* and *MRGD* genes. Interestingly, this interruption time was insufficient for clearance of BEC tissue AA+PP modulation effect, which, although not actually characterizing a typical wash-out, still suggests a certain extended protective effect of the AA+PP formulation. These results may be better clarified in future studies.

On the other hand, *ADRB2* gene jumped back to over-expression in response to medication interruption. This can be explained by the short-term control of the typical β-adrenergic responses of the sympathetic reflex system, through reductions in plasma PP concentrations due to the interruption of the medication. This would be an interesting finding, showing that even the modulation of beta-adrenergic receptor gene has a rapid response, both in the treatment and in the interruption of the treatment. Differently, the RAS is classically known as a tissue and systemic system of medium and long-term endocrine regulation and modulation of local fluid electrolyte balances and controlling vascular haemodynamics.

The classical prohypertensive ACE-AngII-AT1 axis participates in the mediation of pro-inflammatory, prothrombotic and profibrotic processes through activation of the AT1 receptor. In pathological conditions, the ACE-AngII-AT1 axis relates to deleterious effects of anomalous vasoconstriction, inflammation, fibrosis, cell migration, hypertrophy, and fluid retention. An alternative ACE2-Ang(1–7)-MAS counter-regulatory axis shows opposite effects to those produced by ACE-AngII-AT1. ACE2-Ang(1–7)-MAS and is anti-inflammatory, anti-fibrogenic and anti-proliferative, negatively modulating the expression of inflammatory cytokines, such as: TNF-α, IL-1β, IL-6, MCP-1, TGF-β, in addition to reducing the migration and pro-inflammatory function of leukocytes in pathological states [14,15].

Additionally, Lautner et al. [16] have discovered a third ACE2-alamandin-MRGD axis, also counter-regulatory to the ACE-AngII-AT1 axis. These complex axes, once well researched, become important targets for understanding and formulating specific anti-inflammatory drugs. Furthermore, the biochemical and molecular pathways of action of these axes converge with classical pathways of FOP pathophysiology, especially the MAPK and p38 pathways. The ACE-AngII-AT1 axis acts through the NF-kB pathway, activating pro-inflammatory pathways and the development of cytokine storms in exacerbated inflammatory crises [12]. The anti-inflammatory effect of PP, through inhibition of the release of pro-inflammatory cytokines contributes to mitigating the progression of derangement in the expression of inflammatory cytokines. PP interrupts the sympathetic system and cytokine storms interaction through inhibition of its several mediators [12]. Taking advantage of this context, it can be inferred that a cytokine storm should occur in inflammatory crises of FOP. However, this is difficult to monitor during flare-ups, due to the risk of causing further harm to the patient when trying to collect biological material for this evaluation.

The authors conjecture that AA+PP down-regulation of *AGTR1* and *AGTR2* receptors observed in BEC may be due to the putative inhibition of their precursors ACE and AngII, favouring the alternative ACE2-Ang(1–7)-MAS axis, which tends to remain stable during treatment and when treatment is discontinued. Therefore, these data require further studies. Another hypothesis would be the possibility of simultaneous regulation of these genes by the haeterometric physical and functional interdependence of AngII and Ang(1–7) receptors, knowing that, although activated by different agonists, AT2R and MasR are capable of dimerizing and thus sharing signaling pathways [17].

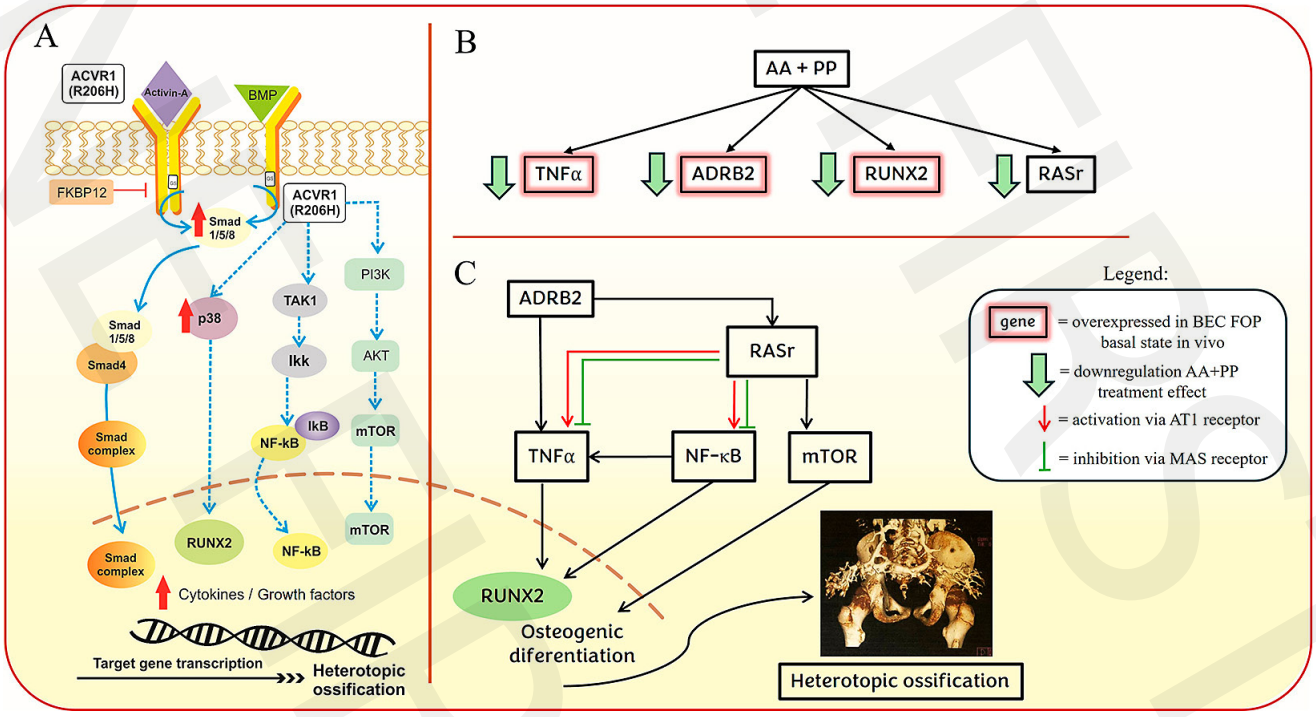


Figure 3. Classical view of target pathways for understanding the pathophysiology of FOP and for therapeutic searches. (A) Canonical biochemical-molecular pathways related to the predominant mutation of the ACVR1 receptor (R206H) linked to pathophysiological consequences. (B) Modulation of AA+PP on putative genes related to convergent inflammatory pathways. (C) Hypothetical scheme of cross-talk between inflammatory pathways with plausible inflammatory-osteogenic fate, speculated as non-canonical therapeutic targets for FOP. (O.B.S) The figure represents typical heterotopic ossification of one of our patients (own file with authorization)

An explanatory hypothesis for the AA+PP effect of double down-regulation of receptors from opposing RAS axes would fit in evidences of physical interaction and functional crosstalk between AT1R and AT2R, independent of the Ang II ligand, in which the heterodimerization of the latter with the former is shown to be capable of inhibiting AT1R function, even without AT2R activation/signaling. On the other hand, AngII/AT1R signaling can be inhibited by constitutive MasR-AT1R heterodimerization, and it is possible that there is an oligomeric complex of native MasR-AT1R-AT2R, whose experimental evidence with functional implications is beginning to appear but still needs to be further studied and clarified [18].

From a pharmacodynamic point of view, in vitro experiments in HEK-293 cells, AA dose-dependently modified the binding properties of the AT1 receptor, reducing its binding affinity, without altering its expression levels on the cell surface, and without affecting the binding properties of AT2R [19]. In vivo treatment with AA led to a reduction in ACE1 expression (mRNA and protein) in the renal cortex of an animal model of hypertension, without altering the expression of AngII type 1 receptors (20). In vitro (PBMC) experiments by the authors showed that AA alone was also unable to modulate *AGTR1* expression, while its association with PP caused the down-regulation of both AngII receptors [7], an effect corroborated here, in vivo in BEC, suggesting that the effect of AA+PP on AngII receptors is obtained by the action of PP rather than AA.

Interestingly, it appears that AT1 receptors interact directly with both subtypes of beta-adrenergic receptors (βAR), so that βAR blockade impairs (or inhibits) AT1 receptor signaling and vice versa, through the functional uncoupling of the reciprocal receptor to its cognate G protein [21]. Moreover, the use of PP in the treatment of vascular diseases, dependent on angiogenesis and cell proliferation, has highlighted the relevant role of the beta-adrenergic system in the expression of essential components of the RAS. For example, Dornhoffer et al. [22] showed a reduction in the expression of AGTR1 and ACE in infantile haemangioma (IH) tissue after treatment with PP. Therefore, in not ignoring the contextual differences between IH and FOP, the authors of the current study infer that the down-regulation of *AGTR1* and *AGTR2* observed here reflects a reduction in AngII activity, so that at the tissue level, AA+PP could contribute to the inhibition of angiogenesis and cell proliferation.

The down-regulation of *AGTR1*, *AGTR2* and *MRGD* may also be associated with the relief of chronic pain, previously observed with the use of AA+PP in FOP patients [9]. Preclinical evidence suggests that Ang II via the AT1 receptor is capable of increasing the nociceptive sensation by acting on neuro medullary components that control pain. Furthermore, while AT2R activation would be capable of facilitating neuronal protection and regeneration in conditions where such processes are pathologically impaired, its antagonism (EMA401) showed an anti-nociceptive effect by inhibiting calcium influx [15].

Also, context-dependent, the interaction of the MRGD receptor with its ligand alamandine is generally attributed protective effects on the cardiovascular and respiratory systems [16,17]. However, with predominant expression in peripheral sensory neurons sensitive to temperature changes and mechanical stimuli, its activation has been associated with inflammatory hyperalgesia via NF-κB signaling [23]. In contrast, increasing MasR signaling – by activating ACE2 and/or increasing Ang(1–7) availability could offer an additional approach to achieving analgesia by counter-regulating AngII/AT1R-induced nociceptive behaviour [15]. Previous in vitro results showed favouritism of the protective ACE2-Ang(1–7)-Mas axis in response to AA+PP [7].

Modulations of RAS components may impact potentially inflammatory, angiogenic, and osteogenic pathways and factors in the context of FOP (Fig. 3). Lan et al. [23] showed MRGD as a positive regulator of canonical NF-κB activation through interaction with TAK1 and the IKK complex in mouse dorsal root ganglia. The NF-κB pathway can also be regulated and activated by AngII via its receptors, enhancing inflammation when signaled by AT1R, through the up-regulation of pro-inflammatory cytokines, such as IL-6 and TNF-α [24]. Interestingly, this pathway, signaled in macrophages, influences several stages of endochondral OH, from the initial inflammatory stage to the differentiation of mesenchymal progenitor cells into chondrocytes, and from these into bone cells [25].

Finally, the role of MAS and MRGD receptor signaling in downstream inhibition of the PI3K/AKT/mTOR pathway (14), with possible impairment in the modulation of chondrogenesis and ectopic bone formation (6), still needs to be clarified. Therefore, it is important to investigate and relate the gene modulations of RAS in response to the use of AA+PP with signaling pathways directly involved in crucial stages of OH, and to clarify its therapeutic effect in controlling exacerbated inflammation and pathological vascular and bone remodeling, without harming physiological processes and homeostasis.

In conclusion, the results in BEC imply that AA+PP can modulate genes in FOP non-canonical physiopathology pathways related to inflammation, cell signaling and osteogenesis processes. The Renin Angiotensin System is certainly a potential target of therapeutic study that may benefit fibrodysplasia ossificans progressiva, needing further investigation aimed at the future use of its anti-inflammatory peptides.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

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