

# Iron overload causes osteoporosis in thalassemia major patients through interaction with transient receptor potential vanilloid type 1 (TRPV1) channels

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Manuscript received on January 27, 2014. Manuscript accepted September 10, 2014.  
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## **Supplement 1: Detailed methods and supplementary figures**

**Human cell cultures** OCs were differentiated from peripheral blood mononuclear cells as previously described<sup>1, 2</sup>. Seeding density was:  $4 \times 10^7$  cells for biomolecular experiments;  $3 \times 10^6$  cells for immunocytochemical and calcium assay experiments; and  $5 \times 10^5$  cells for the Tartrate-resistant acid phosphatase (TRAP) assay experiments.

**RT-PCR and Real Time quantitative PCR** Extraction of mRNA was performed using an RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH), according to the manufacturer's instructions. RNA concentrations were determined by UV spectrophotometer (NanoDrop ND 1000, NanoDrop Technologies, LLC, Wilmington, USA). Reverse transcriptase using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA) was performed. TRAP (alias ACPS, GeneID54), Cathepsin K (alias CTSK, GeneID1513), TRPV1 (transcript variants 1 and 3) (GeneID7442), CB1 (isoforms a and b) (alias CNR1, GeneID1268), CB2 (alias CNR2 GeneID1269), Fatty Acid Amide Hydrolase (FAAH) (GeneID2166), N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (GeneID222236), Diacylglycerol lipase alpha (DAGL- $\alpha$ ) (GeneID221955), Monoacylglycerol lipase (MAGL) (alias MGLL, GeneID11343) and Cannabinoid Receptor Interacting Protein (CRIP1A) (alias CNRIP1 GeneID25927) and the housekeeping  $\beta$ -actin expression levels were analysed. Amplimers were resolved into 2.0% agarose gel, detected by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA, USA) and verified by sequencing using the Big-Dye Terminators reaction kit and an ABI PRISM 310 (Applied Biosystem, Forster City, USA).

In order to quantify the expression levels of TRAP and Cathepsin K, CB1, CB2 and TRPV1 in respect to  $\beta$ -actin, three serial 5x cDNA dilutions obtained from 50 ng and 250 ng total mRNA were amplified by Real-time PCR on ABI PRISM 7900HT

(Applied Biosystem, Forster City, USA), by using Sybr green as fluorophore. 25 $\mu$ l reaction contained: 2 $\mu$ l cDNA, 12.5 $\mu$ l Sybr green Master Mix (BioRad, Berkeley, California, USA) (Biorad), 10 $\mu$ l primers mix (10mM). The thermal cycling program was: 95°C-10min, followed by 40 cycles of 95°C-15s and 60°C-1min. Real-time products were analysed by using the comparative cycle threshold method of relative quantization to the housekeeping gene with ABI PRISM 7900HT Sequence Detection System software (Applied Biosystem, Forster City, USA). All the assays were performed at least in triplicate.

**Tartrate Resistant Alkaline Phosphatase assay** The ACP method (Takara Bio, Japan) was used as previously described(1, 2). TRAP(+)-multinucleated-OCs were counted in at least three different wells *in* each group of treatment through an optical microscope (Nikon Eclipse TS100, Nikon Instruments, Badhoevedorp, Netherlands).

**Immunofluorescence** OCs were incubated for 3h with goat-polyclonal anti-TRPV1 (1:50) and rabbit-polyclonal anti-TRAP (1:100) (Santa Cruz Biotechnology, CA, USA) and mouse-polyclonal anti-vimentine (1:1000; Abcam, Cambridge, UK). Donkey anti-rabbit-IgG-conjugated-AlexaFluorTM568, or anti-goat-IgG-conjugated-AlexaFluorTM488, or anti-mouse-IgG-conjugated-AlexaFluorTM350 were used as the secondary antibody (1:1000; Molecular Probes, USA).

**Western Blot** TRPV1 channels in total lysates from osteoclast cultures were analyzed by Western blot experiments. Membrane strips were incubated overnight at 4 °C with goat polyclonal anti-TRPV1 antibody (1:200 dilution; sc-12502, Santa Cruz, CA, USA); reactive bands were detected by chemiluminescent HRP substrate (Immobilion Western, Millipore, USA) and captured by X-ray film (Fujifilm Corporation, Tokyo, Japan). An anti- $\beta$ -actin (1:5000; Sigma, Milan, Italy) was used to check for identical

protein loading. Images were captured, stored, and analyzed with the Quantity One software (BioRad, Berkeley, California, USA).

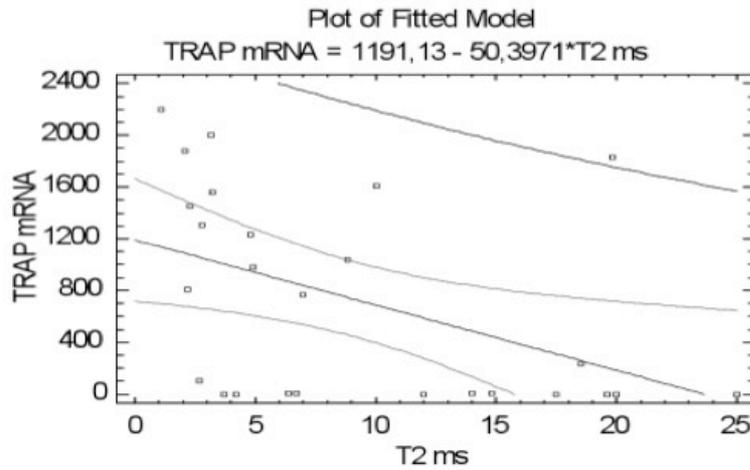
**Drugs and treatments** Resiniferatoxin (RTX) was dissolved in PBS-0.01% DMSO. DFO (deferroxamine mesylate, Desferal, Novartis Pharmaceuticals, Basel, Switzerland), DFP (Ferriprox, Apotex Europe, Leiden, Netherland) and DFX (Exjade, Novartis Pharmaceuticals, Basel, Switzerland) were dissolved in sterile water. OCs were treated with RTX [5 $\mu$ M] for 48 hours after day 21 (full differentiation) or differentiated in the presence of DFO [5mM], [1mM], [100 $\mu$ M], [10 $\mu$ M], from the first day of culture until they were fully differentiated in OCs. Among these concentrations, DFO 10 $\mu$ M was chosen because of the cytotoxicity of higher concentrations. To allow for better comparison of the effects of different iron chelating drugs, we also performed the same experiments using DFP [20 $\mu$ M] and DFX [5 $\mu$ M], replicating therapeutic concentrations. To highlight the role of iron overload, OCs derived from healthy subjects were treated with ammonium iron (III) citrate, and FAC [50  $\mu$ M], (*Sigma-Aldrich* Corporation, Saint Louis, USA) from day 7 (second medium change) until they were fully differentiated into OCs (day 21). They were then either treated with DFO [10 $\mu$ M], DFP [20 $\mu$ M], DFX [5 $\mu$ M] or vehicle (water) from day 15.

RNA extraction or TRAP assay was performed 48h after RTX treatment or on the 22<sup>nd</sup> day.

**Endocannabinoid measurements** OCs were homogenized in chloroform/methanol/TRIS-HCl 50 mM pH 7.4 (2:1:1, v/v) containing 10 pmol of [<sup>2</sup>H]<sub>8</sub>-AEA, [<sup>2</sup>H]<sub>4</sub>- palmitoylethanolamide, (PEA) and [<sup>2</sup>H]<sub>4</sub>-oleoylethanolamide (OEA), and [<sup>2</sup>H]<sub>5</sub>-2-AG as internal deuterated standards (Cayman Chemicals, Ann Arbor, MI). The extract was purified and the eluted fraction containing AEA and 2-

AG analysed as previously described<sup>3, 4</sup>. Analyses were carried out in the ion-monitoring mode using m/z values of 356 and 348, 304 and 300, 330 and 326, and 384.35 and 379.35 (molecular ions +1) for deuterated and undeuterated AEA, PEA, OEA, 2-AG, respectively. Concentrations were calculated by isotope dilution and expressed as pmol per mg of wet tissue.

**Statistics** Molecular data are shown as mean±SD. Cell counting data are shown as mean±SEM. Differences in the mean values were evaluated by an unpaired Student's t-test. To reveal associations between quantitative variables a linear regression was performed. All the statistical analysis was performed using Statgraphics CENTURION XV.II (Adalta, Arezzo, Italy; STATPOINT TECHNOLOGIES INC., Virginia, USA). A *p*-value less than 0.05 was considered statistically significant.



**Coefficients**

Parameter	Least Squares Estimate	Standard Error	T Statistic	p-Value
Intercept	1191,13	229,505	5,19	0,0000
Slope	-50,3971	19,9224	-2,52968	0,0184

**Analysis of Variance**

Source	Sum of Squares	df	Mean Square	F-Ratio	p-Value
Model	3,26512E6	1	3,26512E6	6,40	0,0184
Residual	1,22456E7	24	510234		
Total (Corr.)	1,55107E7	25			

**Supplementary Figure S1**

**TRAP levels directly correlate with liver iron concentration using MRI T2\***

Figures show the regression analysis performed using StatGraph software between MRI T2\* values expressed as milliseconds with TRAP mRNA levels in 25 out of 29 patients.

### Supplementary Table S1

#### Patients were free of parathyroid disease or sex hormone abnormalities

Table summarising levels of 17 $\beta$ -estradiol, progesterone, testosterone, vitamin D and parathyroid hormone in our participants; all of which fell within the normal range. No patients were taking steroid or estrogenic treatment.

	<b>Thalassemic Females (n=20)</b>	<b>Thalassemic Males (n=9)</b>	<b>Thalassemic Patients (n=29)</b>
<b>17<math>\beta</math>-estradiol</b> (pg/ml)	93.1 $\pm$ 55.98 (range 38-200)	—	—
<b>Progesterone</b> (UI/L)	0.49 $\pm$ 0.35 (range 0.07-1.2)	—	—
<b>Testosterone</b> (ng/dl)	—	807 $\pm$ 185.96 (range 457-1040)	—
<b>Vitamin D</b> (ng/ml)	—	—	47.95 $\pm$ 13.03 (range 30.5-78)
<b>PTH</b> (pg/ml)	—	—	20.86 $\pm$ 9.98 (range 6.3-38.5)

## Supplemental References

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