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Iron overload causes osteoporosis in Thalassemia Major patients through interaction with TRPV1 channels

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Abstract

The pathogenesis of bone resorption in β-Thalassemia Major is multifactorial and our understanding of the underlying molecular and cellular mechanisms remains incomplete. Considering the emerging importance of the endocannabinoid/endovanilloid system in bone metabolism, examining a potential role for this system in the development of osteoporosis in β-Thalassemia Major and its relationship with iron overload and iron chelation therapy is warranted. This study demonstrates that, in thalassemic-derived osteoclasts, Tartrate-resistant Acid Phosphatase expression inversely correlates with femoral and lumbar bone mineral density, and directly correlates with ferritin levels and liver iron concentration. The vanilloid agonist resiniferatoxin dramatically reduces cathepsin K levels and osteoclast numbers in vitro, without affecting Tartrate-resistant Acid Phosphatase expression. The iron chelators deferoxamine, deferiprone and deferasirox decrease both Tartrate-resistant Acid Phosphatase and cathepsin K expression, as well as
osteoclast activity. Taken together, these data show that Transient Receptor Potential Vanilloid type 1 activation/desensitization influence Tartrate-resistant Acid Phosphatase expression and activity, and this is dependent on iron, suggesting a pivotal role for iron overload in the dysregulation of bone metabolism in Thalassemia Major patients. Our applied pharmacology provides evidence for the potential of iron chelators to abrogate these effects by reducing osteoclast activity. Whether iron chelation therapy is capable of restoring bone health in humans requires further study, but the potential to provide dual benefits for patients with β-Thalassemia Major, in respect to preventing iron-overload and alleviating associated osteoporotic changes is exciting indeed.

Introduction

Osteopenia and osteoporosis (OP) are responsible for substantial morbidity in adult patients with beta-Thalassemia Major (TM), conferring a significant increase in fracture risk\textsuperscript{1-4} and effecting 40-50% of this population. The pathogenesis of OP is multifactorial and includes environmental (diet and lifestyle), iatrogenic (drugs), acquired (bone marrow expansion, hemochromatosis, hepatitis, deficiency of growth hormone or insulin growth factor I, and hypogonadism) and genetic factors\textsuperscript{1-7}. The relative contribution of these factors to TM-osteoporosis is uncertain, and the role of iron overload and iron chelation therapy is the subject of increasing interest. Interestingly, reports have shown differential responses to bisphosphonate treatment between patients with TM and thalassemia intermedia: in a prospective study, individuals with TM were shown to have high turnover bone disease and responded more favorably to treatment with pamidronate and hormone replacement therapy, as compared with patients with thalassemia intermedia\textsuperscript{8}. The pathological mechanisms
of disturbed bone integrity may therefore be different between TM and thalassemia intermedia. Iron deposition in bone impairs osteoid maturation, inhibits mineralization, and reduces the bone metabolism unit tensile strength, resulting in focal osteomalacia. Three iron chelation therapies (ICT) have been approved for clinical use: deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX). DFO inhibits DNA synthesis, osteoblast and fibroblast proliferation, collagen formation, and enhances osteoblast apoptosis. The effect of DFP and DFX on bone metabolism and health has not been studied.

Even after restoring hemoglobin levels, adequate hormone replacement, and effective iron chelation with normalization of iron status, TM patients continue to show imbalanced bone turnover, with an increased resorptive phase resulting in seriously diminished bone mineral density (BMD). The increased osteoclast activity seems to be at least partially a consequence of an imbalance in the receptor–activator of the nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG) system, and the overproduction of cytokines involved in osteoclast differentiation and function. However, no correlation has been found between RANKL or OPG levels and BMD of the lumbar spine or the femoral neck. Conversely, we have shown a direct correlation between BMD and expression levels of the Tartrate-resistant Acid Phosphatase (TRAP). Interestingly, TRAP is an iron-phosphoesterase and its activation is associated with the redox state of the di-iron metal center or the proteolytic cleavage in an exposed loop domain, due to the cysteine proteinase cathepsin K. Both TRAP and cathepsin K are considered markers of osteoclast activity. The endocannabinoid/endovanilloid system has recently been considered a potential therapeutic target for bone disease. We have previously reported that
human osteoclasts (OCs) express the functional Transient Receptor Potential Vanilloid type 1 (TRPV1) channel together with the cannabinoid receptors type 1 and 2 (CB1/CB2), and metabolic enzymes for the two most studied endocannabinoids; anandamide (AEA) and 2-arachydonoylglycerol (2-AG). Cannabinoid/vanilloid agonists alone, or in combination with selective antagonists, are able to modulate osteoclast formation and activity\textsuperscript{22, 30}. Consistent with data from rodents, which suggest that this system may represent a novel therapeutic target for treating diseases characterized by abnormal bone resorption\textsuperscript{31}, we have also previously shown that expression of TRPV1 and of CB1 vs. CB2 are dramatically modified in OCs from osteoporotic post-menopausal women\textsuperscript{22}. Therefore, when considering the emerging role of the endocannabinoid/endovanilloid system in bone metabolism, and specifically in the pathophysiology of OP, this study aimed to evaluate the possible influence of this system in the development of TM-induced OP and specifically its relationship with iron overload and chelation therapy.

**Methods**

**Patients** Twenty-nine β-TM patients (32% male; mean age 32.1±3.9 years) and 40 controls (40% male; mean age 33±4.3 years) attending the Department of Woman, Child and of General and Specialist Surgery at the Second University of Naples (SUN) were recruited. All patients gave informed written consent on entering the study, which was approved by the SUN Ethics Committee, in accordance with the Declaration of Helsinki. None of the patients had thyroid disease, diabetes mellitus, malabsorption or hypoparathyroidism (see Table S1). All patients received regular transfusions, maintaining pretransfusion hemoglobin levels at approximately 9.5 g/dl. Twenty-four patients had been splenectomised. None of the participants had received
bisphosphonates or steroid/estrogenic treatments for at least three years prior to recruitment.

Iron overload was assessed by measurement of mean serum ferritin levels over the last ten years, and estimation of liver iron concentration (LIC) by T2* magnetic resonance imaging (MRI). The LIC was calculated from liver T2* images (TE 0.99 – 16.50 ms) using the formula previously described \( \frac{1}{[T2^*/1000]} \times 0.0254 + 0.202^{32} \). LIC results were expressed as milligram of iron per gram of liver (dry weight) and 1.8 mg/gr was considered the upper normal limit. Magnetic resonance imaging T2* values were also expressed in milliseconds for additional analysis. The BMD of the lumbar spine (L1–L4) and the femoral neck was determined by Dual Energy X-Ray Absorptiometry (DXA) using Hologic bone densitometer (Hologic, QDR 4500, Hologic Inc., Bedford, MA, USA). Normal (Z-score between 0 and 1), osteopenic (Z-score between -1 and -2.5) and osteoporotic (Z-score below -2.5) groups were defined using the standard World Health Organization criteria\(^{33}\). According to these criteria, 14 patients showed osteoporosis, 9 showed osteopenia and 6 had a normal BMD. Laboratory methods are summarised below and detailed in Supplement 1.

**Human cell cultures** OCs were differentiated from peripheral blood mononuclear cells as previously described\(^{32,30}\). Control osteoclast cultures were harvested in the presence of 10% serum from thalassemic patients (TS), instead of 10% FBS.

**RT–PCR and Real Time quantitative PCR.** TRAP, Cathepsin K, TRPV1 (transcript variants 1 and 3), CB1 (isoforms a and b), CB2, Fatty Acid Amide Hydrolase (FAAH), N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), Diacylglycerol lipase alpha (DAGL-\( \alpha \)), Monoacylglycerol lipase (MAGL),
Cannabinoid Receptor Interacting Protein 1a and the housekeeping β-actin expression levels were analysed using real time PCR and quantitative PCR.

**Tartrate Resistant Alkaline Phosphatase assay.** The ACP method was used as previously described\textsuperscript{22, 30}. TRAP(+) multinucleated-OCs were counted in at least three different wells *in* each group of treatment through an optical microscope.

**Western Blot and Immunofluorescence** TRPV1 channels in total lysates from osteoclast cultures were analyzed by Western blot experiments. Immunofluorescence was used to identify TRPV1, TRAP, and vimentine.

**Drugs and treatments** OCs were treated with Resiniferatoxin (RTX) [5µM] for 48 hours after day 21 (full differentiation) or differentiated in the presence of DFO [10µM], DFP [20µM] or DFX [5µM] from the first day of culture until they were fully differentiated in OCs. Osteoclasts derived from healthy subjects were treated with ammonium iron (III) citrate, FAC [50 µM], from day 7 (second medium change) until they were fully differentiated into OCs (day 21). They were then either treated with DFO [10µM], DFP [20µM], DFX [5µM] or vehicle (water) from day 15. Control OCs were harvested in 10% TS medium from day 1, and either treated or not treated with DFO [10µM], DFX [5µM] or vehicle (water) from day 15. RNA extraction or TRAP assay was performed 48h after RTX treatment or on the 22nd 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 [\textsuperscript{3}H]\textsubscript{8}-AEA, [\textsuperscript{3}H]\textsubscript{4}-palmitoylethanolamide, (PEA) and [\textsuperscript{3}H]\textsubscript{4}-oleoylethanolamide (OEA), and [\textsuperscript{3}H]\textsubscript{5}-2-AG as internal deuterated standards. The extract was purified and the eluted fraction containing AEA and 2-AG analysed as previously described\textsuperscript{34, 35}. 

Results

TRAP is up-regulated in Thalassemic OCs and directly correlates with bone loss

RT-PCR analysis for the osteoclast markers TRAP and Cathepsin K showed that cells in culture were OCs. As expected, both the enzymes TRAP and Cathepsin K were over-expressed in OCs derived from TM patients with respect to healthy controls (Figure 1A).

Interestingly, ferritin and LIC in TM subjects were directly associated with TRAP expression. Simple regression between ferritin or LIC and TRAP mRNA levels were statistically significant (p=0.0002 and p=0.0017, respectively) (Figure 1B). Regression analysis was also performed between MRI T2* values expressed as milliseconds with TRAP mRNA levels in 25 out of 29 patients (Figure S1), to confirm correlations remained statistically significant (p=0.0184). Moreover, TRAP levels inversely correlated with lumbar and femoral BMD (p=0.0347 and p=0.0292, respectively) (Figure 1C).

TRPV1 channels, CB1/CB2 receptors, FAAH, NAPE-PLD, DAGL-α and MAGL mRNAs are expressed in TM-OCs

We confirmed the presence of mature mRNA for TRPV1 and CB1/CB2 receptors, as well as for the endocannabinoid/endovanilloid metabolic enzymes; NAPE-PLD, FAAH, DAGL-α and MAGL (Figure 2A). Although both the CB1 long and short isoforms, namely CB1a and CB1b, were expressed, CB1b levels were higher than those observed in OCs derived from control subjects. A lower expression of CB2 was found in OCs from TM patients as compared to OCs from healthy subjects. TRPV1 expression was significantly up-regulated in TM OCs, with a higher expression of the variant 1 (VR1-1) relative to variant 3 (VR1-3) (Figure 2A). Both the enzymes for
the synthesis and the catabolism of anandamide, NAPE-PLD and FAAH respectively, and for the synthesis of 2-AG and DAGLα, were significantly decreased in TM patients, whereas the enzyme for the degradation of 2-AG and MAGL, were significantly increased (Figure 2A). Accordingly, we found significantly lower levels of both AEA and 2-AG in OCs from TM patients (Figure 2B). We also measured the levels of the two AEA homologues, OEA and PEA, which with AEA share the ability to activate TRPV1. Significantly, the levels of OEA were decreased, whilst those of PEA were increased in OCs from TM patients (Figure 2B).

**TRPV1 channel stimulation decreases Cathepsin K without modifying TRAP and increases CB1/CB2 receptors and TRPV1 channel expression**

Treatment with the selective TRPV1 channel agonist RTX [5 μM] did not significantly affect TRAP mRNA levels in TM OCs. Nevertheless, RTX significantly reduced Cathepsin K mRNA levels in the cells (Figure 3A).

Treatment of TM derived OCs with RTX significantly increased the expression of CB1, CB2 and the cannabinoid receptor interacting protein 1a mRNA levels. In addition, RTX also enhanced the expression of TRPV1 variant 3 (mainly expressed in healthy OCs), with no significant change in the expression of variant 1. RTX also changed AEA metabolic enzymes, significantly reducing FAAH and increasing NAPE-PLD, but with no significant effects on 2-AG enzymes, DAGL or MAGL (Figure 3B).

**TRPV1 stimulation decreases TRAP(+) OC number**

We performed TRAP(+) cell counts on osteoclast cultures from TM OP and non-OP/osteopenic TM patients. Treatment with RTX [5 μM] decreased the TRAP(+)
multinucleated (n≥3) OCs in TM OP patients, whereas no effect was detected for the other TM subjects. Moreover, we observed a reduction in number and activity of OCs in both groups when the cells were treated with the TRPV1 antagonist Iodio-Resiniferatoxin (I-RTX) [2.5μM], suggesting that the effect for the agonist described above was due to activation and subsequent desensitization of the channel (Figure 3C).

Immunocytochemistry shows increased expression of TRPV1 channels in TM activated OCs

Immunolabelling of the TRPV1 channel revealed increased expression in TM OP patients as compared with OCs derived from non-OP TM subjects. The vimentine staining also revealed an increased number of giant and multinucleated osteoclasts in TM-derived plates (Figure 4A). The mean number of nuclei in OCs was higher in TM OP than in TM non-OP subjects (16±6 vs. 5±2). Western blot analysis from total osteoclast lysate demonstrated that OP TM-derived OCs showed a significant increase in TRPV1 protein expression (Figure 4B and C).

Deferoxamine affects the expression of TRAP and Cathepsin K and TRAP(+) OC number in OP TM derived OCs

Osteoporotic TM-derived cell cultures were treated with DFO [10μM]. Both the biomarkers TRAP and cathepsin K were reduced in OCs exposed to DFO, compared with vehicle treated OCs (Figure 5A). Moreover, DFO significantly reduced the number of TRAP(+) multinucleated (n≥3) cells, as revealed by the TRAP assay (t=4.280  p=0.002), and abolished the capability of RTX [5 μM] to significantly reduce the number of OCs (Figure 5B). Of note, DFO showed a cytotoxic effect and
no more than 30% of OCs (all TRAP-positive) survived at the dose chosen in this study.

**OC activity and number are affected by different ICTs**

TRAP assays showed that DFO [10 μM] was able to induce approximately 70-80% reduction in OC activity (Figure 6A), although this effect occurred in parallel with induction of cell death (Figure 6B). Treatment with comparable doses of the two orally active chelators, DFP 20μM and DFX 5μM, showed a significantly lower cytotoxic effect (approximately 55% and 45% of OCs surviving, respectively) compared to DFO (t=-2.824 p=0.011 and t=-2.300 p=0.034, respectively) (Figure 6B). Interestingly, while DFP reduced TRAP(+) OC numbers in a comparable manner to DFO (60% vs. 70%; p>0.05), DFX reduced OC activity significantly more when compared with either DFO (90% vs. 70%, t=-2.655 p=0.016) or DFP (90 % vs. 60%, t=3.473 p=0.003) (Figure 6A).

**Iron overload of healthy derived OCs increases TRAP expression and activity**

Molecular levels of TRAP, revealed by real time PCR in osteoclast cultures derived from healthy subjects, were significantly increased (approximately 25 fold) after FAC-induced iron overload. The co-application from day 15 of DFO [10μM], DFP [20μM] or DFX [5μM], fully reversed this effect. Whereas DFO-treated iron-overloaded OCs expressed higher TRAP levels than vehicle treated cells, DFX and DFP completely abolished FAC-induced TRAP over-expression, and decreased TRAP levels with respect to vehicle. However, only treatment with DFX showed a significant effect (90% reduction vs. vehicle) (Figure 7A).
TRAP assays revealed that *in vitro* iron-overload using FAC [50 μM], induced an increase in both osteoclast number (more than 40%) and size. The co-treatment from day 15 with DFO 10μM, DFP 20μM or DFX 5μM, fully reversed this effect, with DFX demonstrating the largest effect (10%, 60% and 70%, respectively) (Figure 7B).

**Serum from TM patients increases number of TRAP(+) OCs from healthy controls**

*In-vitro* iron overload of OCs from healthy subjects using serum from TM patients induced an increase in TRAP(+) cell number (Figure 8). This increase in percentage of active OCs was significant (TS vs control, t=3.453, n=3, p=0.03) and was able to be significantly reversed following treatment with DFX, but not with DFO (TS-DFX vs TS t=-4.079, n=3, p=0.015; TS-DFO vs TS t=-1.88, n=3, p=0.132). Moreover, control-derived osteoclasts treated with chelators showed increased activity (DFO vs control t=1.394, n=3, p=0.05; DFX vs control t=2.465, n=3, p=0.17).

**Discussion**

The molecular and cellular mechanisms responsible for the pathogenesis of bone resorption in TM remain poorly understood. Osteoblast dysfunction is recognised as a critical pathogenetic mechanism, and decreased serum levels of osteocalcin occur in patients with TM. There is also evidence of increased osteoclast activation in TM patients. Accordingly, elevated markers of bone resorption have been found in TM subjects. Endocrinopathies are common in TM patients, and these may also contribute to osteopenia-osteoporosis syndrome, particularly hypogonadotrophic hypogonadism. Similarly, anti-resorption therapies such as bisphosphonates in combination with hormone replacement regimens lead to improvements in bone
integrity in thalassemic patients\textsuperscript{16}. In the interest of examining a homogenous group of patients and limiting the explanatory variables, we recruited patients without thyroid disease, diabetes mellitus, malabsorption, hypoparathyroidism or on steroid/estrogenic treatment (see Table S1). Studies have, however, previously shown that patients with TM show relatively less impressive improvements in spinal bone mineral density following hormone replacement therapy, as compared to individuals with premature ovarian failure, suggesting mechanisms of osteoporosis independent of hypogonadism\textsuperscript{16}.

In this study, we found increased expression of TRAP and cathepsin K mRNA levels in TM-derived osteoclasts, which correlated significantly with reduction in femoral and lumbar BMD (Figure 1). This suggests a role for activated osteoclasts in the pathogenesis of TM-related bone resorption. Interestingly, enhancement of TRAP directly and significantly correlated with ferritin levels and LIC, confirming a key role for iron overload in the pathogenesis of TM-associated bone disease. LIC was calculated from liver T2* images (TE 0.99 – 16.50 ms) using the formula previously described \((1/[T2*/1000]) \times 0.0254 + 0.202\)\textsuperscript{32}. Different MRI parameters have been used to indicate liver iron levels\textsuperscript{32, 38, 39}, specifically including MRI R2 measurements; which have been validated for LIC. Nonetheless, TRAP mRNA did correlate with T2* units independent of the calibration equation in this study (see Figure S2 for regression analysis).

Our data also implicates the endocannabinoid/endovanilloid system in activation of osteoclasts. Osteoclasts from patients with TM express higher levels of CB1 and TRPV1 receptors, as compared with healthy-derived osteoclasts (Figure 2). This is
consistent with previous findings in osteoclasts derived from women with post-menopausal osteoporosis. When considering the profound influence of hormones in growth and remodelling, it would have been interesting to examine gender differences in our study, but the relatively small sample size precludes this.

Previous reports have shown that CB1 and TRPV1 play a cooperative excitatory role in stimulating osteoclast activity, whereas CB2 activation is inhibitory. This is accompanied by a pattern of alterations in endocannabinoid levels – with a reduction in those with highest efficacy at CB2 receptors (2-AG), and an increase in those which activate TRPV1 (PEA). Thus, increased TRPV1/CB1 receptors with decreased CB2 receptor activity may represent one of the molecular events underpinning the development of OP. Consistent with this, we found that CB1b and TRPV1 isoform 1 were strongly over-expressed in OCs from thalassemic patients, whereas the CB1a and TRPV1-3 isoforms were present at lower levels (Figure 2). Although these receptor isoforms seem to exhibit similar pharmacology, they may have different functions in cell physiology. Indeed, the majority of our knowledge of the cannabinoid receptors is in relation to their ligands and receptor binding sites, second messengers or signal transduction mechanisms, and post-receptor intracellular protein-protein interactions. Conversely, little is known about regulation of CB1 or CB2 gene expression. The levels and anatomical distribution of CB1 mRNA and protein are developmental stage-specific and can be dysregulated in several pathological conditions. Moreover, exposure to a variety of challenges may deeply alter CB1 and CB2 gene expression and mRNA levels.
Given the potential anti-osteoporotic effects of TRPV1 pharmacological stimulation / desensitization\textsuperscript{22}, we treated osteoclasts from TM patients with the TRPV1 agonist RTX, which led to an increase in CB2 receptor expression and a dramatic reduction of cathepsin K (Figure 3). This occurred in the absence of any significant change in TRAP mRNA levels (Figure 3A). Treatment with RTX did however, lead to reduced TRAP(+) osteoclasts (Figure 3C). This could potentially be explained if iron overload in TM prevents TRPV1 activation from further modifying TRAP levels - which are directly regulated by iron at the gene transcription level\textsuperscript{48}. Indeed, TRAP is an iron phosphatase, thus the presence of iron overload, which is characteristic of patients with TM, may activate TRAP transcription per se. On the other hand, the activation of TRAP protein is associated with the proteolytic cleavage in an exposed loop domain due to cathepsin K\textsuperscript{23}, which was found to be significantly decreased in RTX treated TM-derived osteoclasts (Figure 3A). Accordingly, we found that iron overload of healthy-derived osteoclasts, obtained by FAC or using thalassemia serum, induced an increase in osteoclast number, size and activity, and increased levels of TRAP. These effects were completely abolished by the chelating agents DFX, DFP and DFO (Figure 7 and 8). It should be noted that the concentrations of FAC used in these experiments was supraphysiological, and likely exceeds the binding capacity of the chelators tested. The abrogation of TRAP levels seen in response to the chelating agents (Figure 7B) may therefore represent alternative mechanisms.

To further support the involvement of iron overload, we differentiated osteoclasts from osteoporotic thalassemic subjects in the presence of the iron chelating agent deferoxamine. In support of the above hypothesis, DFO-treated osteoclasts showed decreased levels of the osteoclast biomarkers; TRAP and cathepsin K (Figure 5).
RTX treatment of DFO-exposed osteoclasts from TM subjects resulted in a slight increase in both osteoclast number and activity; similar to osteoclasts derived from healthy subjects. These data indicate that the capability of TRPV1 activation/desensitization to influence TRAP expression and activity is dependent on iron, suggesting a central role for iron overload in the dysregulation of bone metabolism in TM patients.

In agreement with previous studies of the importance of iron in function of hematopoietic stem cell lineage, we observed a high cytotoxicity in DFO-treated osteoclast cultures. Consequently, we decided to test two other iron chelators, DFP and DFX. Both oral chelators exerted a lower effect on cell viability compared with DFO (p <0.05), and DFX additionally showed stronger effects in term of reduction in osteoclast activity (p<0.05). Although it’s not possible to draw conclusions on the potential efficacy of different chelating agents on preventing TM-associated bone resorption in this experimental in-vitro setting, given the large differences in pharmacokinetics between DFX and DFO (binding 99% and 10% of plasma proteins, respectively), it’s quite possible that differences may exist between these agents in this respect. In our cultures DFX binds to FBS proteins and this link may attenuate DFX cytotoxicity.

In summary, this study provides evidence that osteoclast activity is increased by elevated levels of ferritin and LIC, as demonstrated by biomarker TRAP and cathepsin levels, which together correlate with reduced bone mineral density. The expression of TRPV1 channels, and the positive balance of CB1 vs. CB2 cannabinoid receptors in TM osteoclasts appear to play cooperative role in stimulating osteoclasts.
Indeed, exposing healthy osteoclasts to serum from TM patients was capable of increasing TRAP levels. Pharmacological stimulation of TRPV1 increased activation of osteoclasts in healthy subjects\textsuperscript{30}, whilst TRPV1 antagonism and desensitization can induce over-expression of the protective CB2 cannabinoid receptor - which acts as an “anti-inflammatory” counterpart receptor system in osteoporotic conditions\textsuperscript{22}. Activation in TM subjects did not alter TRAP levels, but there was a signal to reduced levels following treatment with iron chelating agents, suggesting that TRPV1 activation and desensitisation is dependent on iron. Treatment with iron chelators was associated with reduced levels of the osteoclast markers TRAP and cathepsin K.

For the first time, we demonstrate that iron overload, through up-regulation of TRAP expression, causes over-activity of osteoclasts in TM. Normal activity levels can be restored with chelation therapy, opening the possibility that oral chelation therapy may therefore have a role in alleviating TM-associated OP.

**List of abbreviations**

Thalassemia Major (TM); osteoporosis (OP); iron chelation therapy (ICT); deferoxamine (DFO); deferiprone (DFP); deferasirox (DFX); bone mineral density (BMD); the nuclear factor-kappa B ligand (RANKL); osteoprotegerin (OPG);

Tartrate-resistant Acid Phosphatase (TRAP); osteoclasts (OCs); Transient Receptor Potential Vanilloid type 1 (TRPV1); cannabinoid receptor type 1(CB1); cannabinoid receptor type 2 (CB2); anandamide (AEA); 2-arachydonylglycerol (2-AG); liver iron concentration (LIC); magnetic resonance imaging (MRI); Fatty Acid Amide Hydrolase (FAAH); N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD); Diacylglycerol lipase alpha (DAGL-α); Monoacylglycerol lipase (MAGL);
Resiniferatoxin (RTX); $[^2]H_4$-palmitoylethanolamide (PEA); $[^2]H_4$-oleoylethanolamide (OEA); Iodio-Resiniferatoxin (I-RTX).

**Authorship Contributions:** FR, SP, SM and VDM were the principal investigator and takes primary responsibility for the paper; MT and MF recruited the patients; GB, LL, CT and DS performed the laboratory work for this study; GB and LL participated in the statistical analysis; BN and SP co-ordinated the research; FR, SP, SM, GB and LL wrote the paper; all other authors reviewed and contributed their comments to each draft and approved the final version of the manuscript.

**Conflict of Interest Disclosures:** S.P. has received honoraria from Novartis; all other authors declare no potential conflicts of interest.

**REFERENCES**


FIGURE LEGENDS

Figure 1

(A) Osteoclastic markers TRAP and Cathepsin K are up-regulated in thalassemic OCs

The markers of bone resorption; TRAP and Cathepsin K are significantly higher in OCs derived from subjects with TM, as compared with controls. Data have been
acquired from human in vitro OCs by real time PCR normalized for the housekeeping 
β-actin. Data are presented as mean ± SD. One-way ANOVA followed by student-
Neuman-Keuls post hoc test has been used for statistical analysis. p<0.05 was 
considered statistically significant.

(B) TRAP levels directly correlate with ferritin and liver iron concentration
Figures show the regression analysis performed using StatGraph software between 
ferritin and LIC values with TRAP mRNA levels in 25 out of 29 patients.

(C) Lumbar and femoral bone mass loss are directly associated with TRAP 
mRNA levels
Figures shows the regression analysis performed using StatGraph software between 
lumbar and femoral Z-score associated DEXA values with TRAP mRNA levels in 25 
out of 29 patients.

Figure 2
(A) TRPV1 channels, CB1/CB2 receptors, FAAH, NAPE-PLD, DAGLα and 
MAGL mRNAs are expressed in thalassemic human OCs in culture
Data have been revealed from human in vitro OCs by PCR starting from 250 ng of 
total mRNA for the RT reaction and have been normalized for the housekeeping gene 
β-actin. Data are presented as mean ± SD. One-way ANOVA followed by student-
Neuman-Keuls post hoc test has been used for statistical analysis. p<0.05 was 
considered statistically significant. VR1 = TRPV1. 1, 3 = isoform 1, 3.

(B) Measurement of the levels of endocannabinoids (AEA and 2-AG), 
oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) in OCs
OCs from thalassemic patients contained measurable amounts of AEA (0.54±0.17 
pmol/mg extract), 2-AG (2.95±1.26 pmol/mg), and OEA (2.09±0.44 pmol/mg), which
were significantly lower in comparison to those found in control-derived osteoclasts [AEA (1.6±0.23 pmol/mg extract), 2-AG (26.4±5.82 pmol/mg), and OEA (5.7±1.14 pmol/mg)]. Conversely, thalassemic-derived OCs contained higher amounts of PEA (13.38±5.11 pmol/mg), with respect to control-derived OCs [PEA (3.8±0.91 pmol/mg)]. Data are presented as mean±SEM from 4 to 6 experiments.

Figure 3

TRPV1 channel stimulation decreases Cathepsin K without modifying TRAP, increases CB1/CB2 receptors and TRPV1 channel expression, and decreases TRAP(+) OC number

(A) RTX [5μM] significantly reduces the levels of Cathepsin K (CTH K), with no change in TRAP expression levels. (B) RTX [5μM] induces a significant increase in CB1 and CB2 expression and accordingly, an increase of the cannabinoid receptor interacting protein 1A, as well as significant increase in the vanilloid receptor TRPV1, in particular isoform 3. Pharmacological treatment with RTX [5μM] also significantly modulates expression levels of the AEA metabolic enzymes, increasing NAPE-PLD and reducing FAAH, but with no change in the expression levels of the 2-AG metabolic enzymes, DAGL-α and MAGL. Data have been revealed from human in vitro OCs by PCR starting from 250 ng of total mRNA for the RT reaction and have been normalized for the gene β-actin. Data are presented as mean ± SD. One-way ANOVA followed by student-Neuman-Keuls post hoc test has been used for statistical analysis. $p<0.05$ was considered statistically significant. (C) Treatment with the vanilloid agonist RTX [5μM] significantly reduces the number of OCs derived from osteoporotic TM patients but not those derived from non-osteoporotic TM subjects. Treatment with the vanilloid antagonist I-RTX [2.5μM] reduces OC number
in both osteoporotic and non-osteoporotic TM subjects. Data are presented as mean ± SEM. One-way ANOVA followed by student-Neuman-Keuls post hoc test has been used for statistical analysis. \( p<0.05 \) was considered statistically significant.

**Figure 4**

**Immunohistochemistry shows increased expression of TRPV1 channels in TM activated OCs**

(A) Immunolabelling of the TRPV1 channel shows increased expression in TM subjects, in comparison with controls. Vimentine (Vim) staining also revealed an increased number of giant osteoclasts in TM derived plates. Scale = 100\( \mu \)m. (B-C) Western blot analysis for the TRPV1 channel in total OCs lysate, normalized with respect to \( \beta \)-actin, showed a significant increase in TRPV1 protein expression in OP-derived OCs, which was attenuated by treatment with the vanilloid agonist RTX [5\( \mu \)M]. Data are presented as mean ± SD. One-way ANOVA followed by student-Neuman-Keuls post hoc test has been used for statistical analysis. \( p<0.05 \) was considered statistically significant. MW=molecular weight.

**Figure 5**

(A) Deferoxamine affects the expression of TRAP and Cathepsin K in TM OCs

Treatment with deferoxamine (DFO) [10\( \mu \)M] is able to drastically reduce expression levels of both TRAP and Cathepsin K biomarkers. Data were acquired from human in vitro OCs by real time PCR starting from 100 ng of total mRNA for the RT reaction, and have been normalized for the housekeeping gene \( \beta \)-actin. Data are presented as a mean ± SD. One-way ANOVA followed by student-Neuman-Keuls post hoc test has
been used for statistical analysis. \( p < 0.05 \) was considered statistically significant. NT = water or DMSO 0.01%.

(B) Deferoxamine affects TRAP(+) OC number

Deferoxamine (DFO) \([10 \mu M]\) is able to reduce OC number, while treatment of OCs differentiated in the presence of DFO with RTX was not able to reduce the number of TRAP(+) multinucleated \((n>3)\) cells.

Data are presented as a mean ± SD from \( n=6 \) for each group. One-way ANOVA followed by student-Neuman-Keuls post hoc test has been used for statistical analysis. \( p < 0.05 \) was considered statistically significant. NT = water or DMSO 0.01%.

Figure 6

Cytotoxicity and osteoclast activation effects of different iron chelators

(A) Treatment with DFO \([10 \mu M]\), DFP \([20 \mu M]\) and DFX \([5 \mu M]\) reduced numbers of TRAP(+) OCs. The effect exerted by DFX was comparable to that of DFO and significantly higher with respect to DFP. (B) DFO induced reductions in OC activity occurred in parallel with cellular death. Conversely, treatment with DFP \([20 \mu M]\) and DFX \([5 \mu M]\) showed lower cytotoxic effects. The effects on cell viability of these two iron chelating agents did not significantly differ. Data are presented as a mean ± SD from \( n=10 \) for each group. One-way ANOVA followed by student-Neuman-Keuls post hoc test has been used for statistical analysis. \( p < 0.05 \) was considered statistically significant.
Figure 7
Iron overload induced in-vitro affects TRAP expression levels and OC number

(A) FAC-induced iron overload significantly increased the expression levels of TRAP.

The co-application of DFX [5µM], DFP [20µM] or DFO [10µM] reversed this effect.

Data have been revealed from human in vitro OCs by real time PCR starting from 100 ng of total mRNA for the RT reaction and have been normalized for the housekeeping gene β-actin. (B) FAC-induced iron overload increased number and size of TRAP(+) OCs. Co-treatment with DFX 5µM, DFP 20µM or DFO 10µM reversed this effect.

Vehicle = water.

Data are presented as a mean±SD. A t- test has been used for statistical analysis. p<0.05 was considered statistically significant.

Figure 8
Thalassemia sera increases number of TRAP(+) osteoclasts from healthy controls

(A) TRAP assay performed on healthy subjects derived OCs cultures not treated. (B) Serum from thalassemic patients, TS, significantly increases the percentage of TRAP(+) cells (TS vs CTRL n=3, t=3.453, p=0.03). (C) DFO [10µM] reverses the effect induced by TS (TS-DFO vs TS n=3, t=-1.88, p= 0.132). (D) DFX [5µM] significantly reverses the effect induced by TS (TS-DFX vs TS n=3, t= -4.079, p=0.015). (E) DFO [10µM] induces an increase in TRAP(+) cells (DFO vs CTRL n=3, t=1.394, p=0.048). (F) DFX [5µM] induces an increase in TRAP(+) cells with respect to CTRL (DFX vs CTRL n=3, t=2.465, p=0.17. (G) Relative graph showing the percentage of TRAP(+) cells with respect the total cell number for each treatment
(CTRL=10%FBS containing medium; TS=10% thalassemia sera containing medium; DFO=deferoxamine; DFX=desferasirox). Data are presented as a mean±SD. A t-test has been used for statistical analysis. p<0.05 was considered statistically significant.
Supplement 1: Detailed methods and supplementary figures

**Human cell cultures** OCs were differentiated from peripheral blood mononuclear cells as previously described\(^1,2\). Seeding density was: 4x10\(^7\) cells for biomolecular experiments; 3x10\(^6\) cells for immunocytochemical and calcium assay experiments; and 5x10\(^5\) 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. 25µl reaction contained: 2µl cDNA, 12.5µl Sybr green Master Mix (BioRad, Berkeley, California, USA) (Biorad), 10µl 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 HPR substrate (Immobilion Western, Millipore, USA) and captured by X-ray film (Fujifilm Corporation, Tokyo, Japan). An anti-β-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 (deferoxamine mesylate, Desferal, Novartis Pharmaceuticals, Basel, Switzerland), DFP (Ferriprox, Apotex Europe, Leiden, Netherlands) and DFX (Exjade, Novartis Pharmaceuticals, Basel, Switzerland) were dissolved in sterile water. OCs were treated with RTX [5µM] for 48 hours after day 21 (full differentiation) or differentiated in the presence of DFO [5mM], [1mM], [100 µM], [10 µM], from the first day of culture until they were fully differentiated in OCs. Among these concentrations, DFO 10µM 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 [20µM] and DFX [5µM], 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 µM], (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 [10µM], DFP [20µM], DFX [5µM] or vehicle (water) from day 15.

RNA extraction or TRAP assay was performed 48h after RTX treatment or on the 22nd 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 \[^3H\]χ-AEA, \[^3H\]χ-palmitoylethanolamide, (PEA) and \[^2H\]ψ-oleoylethanolamide (OEA), and \[^2H\]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\textsuperscript{3, 4}. 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.
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.

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Analysis of Variance

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Supplementary Table S1

Patients were free of parathyroid disease or sex hormone abnormalities

Table summarising levels of 17β-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.

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<th>Thalassemic Patients (n=29)</th>
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Supplemental References


