HYPOXIA-REPERFUSION AFFECTS OSTEOGENIC LINEAGE AND PROMOTES SICKLE CELL BONE DISEASE

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KEY POINTS
• In sickle cell disease, recurrent vaso-occlusive crisis suppresses osteogenic lineage and activates osteoclasts.
• Zoledronic acid acting on both osteoclast and osteoblast compartment is a multimodal therapy to prevent sickle bone disease.
ABSTRACT

Sickle cell disease (SCD) is a worldwide distributed hereditary red cell disorder, characterized by severe organ complication. Sickle bone disease (SBD) affects the large part of SCD patients and its pathogenesis has been only partially investigated. Here, we studied bone homeostasis in humanized mouse model for SCD. Under normoxia, SCD mice display bone loss and bone impairment with increased osteoclast and reduced osteoblast activity. Hypoxia/reperfusion (H/R) stress, mimicking acute vaso-occlusive crises (VOCs), increased bone turnover, osteoclast activity (RankL) and osteoclast recruitment (Rank) with up-regulation of Il6 as pro-resorptive cytokine. This was associated with further suppression of osteogenic lineage (Runx2, Sparc). In order to interfere with the development of SBD, zoledronic-acid, a potent inhibitor of osteoclast activity/osteoclastogenesis and promoter of osteogenic lineage, was used in H/R exposed mice. Zoledronic-acid markedly inhibited osteoclast activity and recruitment, promoting osteogenic lineage. The recurrent H/R stress further worsened bone structure, increased bone turnover, depressed osteoblastogenesis (Runx2, Sparc) and increased both osteoclast activity (RankL, Cathepsin k) and osteoclast recruitment (Rank) in SCD mice compared to either normoxic or single H/R episode SCD mice. Zoledronic-acid used before recurrent VOCs prevented bone impairment and promoted osteogenic lineage. Our findings support the view that SBD is related to osteoblast impairment and increased osteoclast activity resulted from local hypoxia, oxidative stress and the release of pro-resorptive cytokine such as IL6. Zoledronic acid might act on both osteoclast and osteoblast compartment as multimodal therapy to prevent SBD.
INTRODUCTION

Sickle cell disease (SCD) is a worldwide distributed hereditary red cell disorder, which affects approximately 75,000 individuals in the United States and almost 20,000-25,000 subjects in Europe, this latter mainly related to the immigration fluxes from endemic areas such as Sub-Saharan Africa to European countries. Studies of global burden disease have pointed out the invalidating impact of SCD on patient quality of life. This requires the development of new therapeutic options to treat sickle cell related acute and chronic complications.

SCD is caused by a point mutation in the $\beta$-globin gene resulting in the synthesis of pathological hemoglobin S (HbS). HbS displays peculiar biochemical characteristics, polymerizing when deoxygenated with associated reduction in cell ion and water content (cell dehydration), increased red cell density and further acceleration of HbS polymerization. Pathophysiological studies have shown that dense, dehydrated red cells play a central role in acute and chronic clinical manifestations of SCD, in which intravascular sickling in capillaries and small vessels leads to vaso-occlusion and impaired blood flow with ischemic/reperfusion injury. In microcirculation, vaso-occlusive events (VOC) result from a complex and still partially known scenario, involving the interactions between different cell types, including dense red cells, reticulocytes, abnormally activated endothelial cells, leukocytes, platelets and plasma factors.

Target organs, such as bone or lung, are involved in both acute and chronic clinical manifestations of SCD, related to their peculiar anatomic organization mainly characterized by sluggish circulation and relative local hypoxia. Although progresses have been made in the knowledge of sickle cell related organ damage, much still remains to be investigated in the pathogenesis of sickle bone disease (SBD). Bone imaging studies by magnetic resonance of SCD patients in the early phase of VOCs have documented the presence of abnormal periostal signal intensity, which then develops in areas of bone infarctions. Thus, VOCs combined with marrow hyperplasia and inflammation has been suggested to contribute to the development of SBD. Recently, it has been proposed a possible role of vitamin D deficiency in SBD, which appears to be subordinated to the primary defect in bone homeostasis.

Studies in other models have shown that bone remodeling is a complex process, involving osteoblastic and osteoclastic activity and promoting bone formation/resorption.
To this process, the transcription factor Runx2 plays a pivotal role for osteoblastic differentiation by activating downstream genes such as Sparc (Osteonectin). Whereas, the osteoclastic differentiation and activation is related to osteoclast cell-surface receptor, Rank. 15,16

Here, we demonstrated that SBD results from the combined reduced osteoblast recruitment and increased osteoclast activity. Recurrent hypoxia/reoxygenation (H/R) events, mimicking acute VOCs, activate osteoclastogenesis and bone turn-over. In SCD mice, zoledronic acid ameliorates bone impairment by restraining osteoclast activity in favor of osteoblasts. In a model of recurrent VOCs, zoledronic acid acts on both osteoclast and osteoblast compartment as multimodal therapy, preventing bone loss and bone impairment. Our study for the first time involves osteoblast in SBD and provides the rationale of zoledronic acid as therapeutic intervention to prevent SBD.

**MATERIALS AND METHODS**

**Mouse model and design of the study.** Experiments were performed on healthy control (Hba^tm1(HBA)Tow Hbb^tm3(HBG1,HBB)Tow AA) and SCD (Hba^tm1(HBA)Tow Hbb^tm2(HBG1,HBB*)Tow SS) mice. 8,17 The Institutional Animal Experimental Committee, University of Verona (CIRSAL) and by the Italian Ministry of Health approved the experimental protocols. Sex (M:F ratio 1:1) and aged (16-18 weeks of age) matched mice were studied. 18 Whenever indicated, mice were exposed to hypoxia (8% oxygen for 10 hours) followed by 3 hours reoxygenation (21% oxygen) (H/R stress) to mimic a single acute vaso-occlusive crisis (VOCs) as previously described. 17,19,20 For recurrent VOCs, AA and SS mice were exposed to 3 times VOC (8% oxygen for 10 hours followed by 3 hours reoxygenation, 21% oxygen) with 3 weeks time interval between each VOC.

Hematological parameters, red cell indices and reticulocyte count were determined as previously reported. 21,22 Blood was collected with retro-orbital venipuncture in anesthetized mice using heparined microcapillary tubes. Hematological parameters were evaluated on a Bayer Technicon Analyser ADVIA. Hematocrit and hemoglobin were manually determined. Whenever indicated, zoledronic acid (Zol) was administered at the dosage of 100 ug/Kg intraperitoneally (ip) based on previous reports. 23,24

**Flow cytometric analysis of mouse erythroid precursors of murine bone marrow erythroblasts.** Flow cytometric analysis of erythroid precursors from bone marrow and spleen of mice from the four strains was carried out as previously described using the
CD44-TER-119 strategy.\textsuperscript{25, 26} 

**Measurements of bone homeostasis and turnover.** For details of bone histomorphometry and bone turnover see Supplemental Methods.\textsuperscript{27-30} The number of nodes (branch points) and termini (the endpoints) in a trabecular network, which was skeletonized to facilitate examination of its topological properties, were analyzed according to Garrahan et al.\textsuperscript{31} In a bone section, the ratio of nodes to termini (Nd/Tm) ratio is an index of spatial connectivity. Thus, higher values correspond to better connectivity.\textsuperscript{32} In bone histomorphometry, Marrow Star Volume (MSV) is defined as the mean volume of all parts of an object that can be seen unobscured from a random point within the object.\textsuperscript{33} Higher values of MSV correspond to a reduced connectivity.\textsuperscript{33} To evaluate bone resorption, the Number of Osteoclast/Tissue Area (NOc/TA) and Erosion Surface/Bone Surface (ES/BS), corresponding to bone covered by eroded cavities, were measured according to international guidelines.\textsuperscript{29}

**Colony forming unit-osteoblast (CFU-Ob) assay.** CFU-Ob assay was conducted as previously reported.\textsuperscript{34} Briefly, 1 $\times$ 10\textsuperscript{6} bone marrow nucleated cells from AA and SS mice were seeded in 60-mm culture dishes in osteogenic differentiation medium ($\alpha$-minimum essential medium supplemented with 50 $\mu$g/ml l-ascorbic acid and 2.0 mM $\beta$-glycerophosphate) and incubated at 37°C under humidified atmosphere of 5% CO\textsubscript{2}. The medium was changed every 2 days and after 21 days of culture CFU-Ob colonies were identified by alizarin red staining as previously described.\textsuperscript{34, 35}

**Bone Total RNA extraction and reverse transcription.** Total RNA was extracted from each pellet using the RNeasy minikit (Qiagen, Milano Italy) with DNase I treatment. Tibia bones were harvested from mice and any attached tissue was removed before RNA later solution was added and stored at 4°C. After 24h the RNA later was eliminated and the samples were stored at -80°C until RNA extraction. Frozen samples were crushed using a mortar and pestle in liquid nitrogen as previously described\textsuperscript{36} and homogenized in RLT Lysis buffer. Details are reported in Supplemental Methods. Real Time PCR was performed as previously described.\textsuperscript{37} Details are reported in Supplemental Methods. 

**Serum bone ALP and CTXI evaluation.** Sera were collected and stored at -80° until analyses. The amounts of bone-ALP and CTXI were determined using Mouse Bone-specific Alkaline Phosphatesase (BALP) Elisa Kit (CSB-E11914m; Cusabio) and the CTX –I ELISA kit (CSB-E12782m; Cusabio) respectively. The assays were performed on 96-well immunoplates, according to the procedures recommended by the manufacturers.

**Statistical analysis.** Statistical analyses were performed using SPSS for Windows
version 21.0 (SPSS Inc., Chicago; IL). The 2-way ANOVA algorithm for repeated measures was used for data analysis. Differences with $P<0.05$ were considered significant.

RESULTS

Sickle cell mice show bone structure impairment and increase osteoclast activity

In order to study the pathogenesis of SBD related to the hematological phenotype, we studied humanized sickle cell (SS) mice fed with a standard diet containing 0.97% calcium, 0.85% phosphorus, 1045 U/Kg vitamin D$_3$, 22.5% protein, 5.5% fat, 52% carbohydrate to avoid confounding contribution of vitamin D deficiency on bone homeostasis. SCD mice displayed reduced node number and node/termini ratio with increased marrow star volume compared to control mice expressing normal hemoglobin (AA), suggesting an impairment of bone microarchitecture (Fig. 1A). In SCD mice, we also found erythroid hyperplasia without perturbation of erythroid maturation profile compared to controls similarly to human SCD subjects and in agreement with the increased marrow star volume documented in SCD mice (Fig. 1SA).

Bone volume was significantly decreased in SCD compared to healthy mice (Fig. 1B). The trabecular width and thickness was decreased, while the trabecular separation was increased in SCD mice compared to controls, indicating the presence of bone loss in SCD mice (Fig. 1B). This finding is in agreement with a previous preliminary report in the same mouse model.\textsuperscript{18}

Since bone homeostasis is mainly related to the balance between bone resorption/bone formation, we evaluated osteoblast/osteoclast function in both mouse strains. In SCD mice, we found reduced number of osteoblasts (OBs/BS; Fig.1C; N.Ob/BS Fig. 1SB) associated with decreased osteoid surface (Fig. 1C), indicating a lower osteoblast activity in SCD compared to healthy mice. These findings were in agreement with the down-regulation of molecular osteogenic markers such as Runx2 and Sparc (Osteonectin) in SCD mice (Fig. 1D). Whereas, osteoclastic activity was markedly increased as supported by higher osteoclasts number associated with increased erosion surface in SCD mice compared to controls (Fig. 1C). RankL, a marker of osteoclast activation, was significantly up-regulated in SCD mice compared to healthy controls (Fig. 1D); while, no significant changes in the expression of Rank, a marker of osteoclast recruitment, was observed in SCD mice (Fig. 1D). We also analyzed the expression of Il6, a pro-resorptive
cytokine, which cooperates with RankL on osteoclast activation.\textsuperscript{38,39} As shown in Fig. 1D, Il6 expression was up-regulated in SCD mice compared to controls. These data indicate an increase osteoclast activity combined with possible perturbation of osteoprogenitor compartment in SCD mice. Indeed, we found a decreased ability of mesenchimal stem cells (MSCs) from SCD mice to form osteoblast-CFU compared to healthy controls, suggesting a defect in MSCs recruitment to osteoblasts in SCD mice (Fig. 1E).

Since anti-oxidant systems, such as peroxiredoxin-2 (Prx2) or catalase, have been recently reported to negatively regulate osteoclastogenesis, we analyzed the expression of both anti-oxidant systems in bones from SCD and control mice.\textsuperscript{40,41} Prx2 gene expression was markedly up-regulated in SCD mice compared to healthy controls (Fig. 1SC), while Catalase expression was similar in both mouse strains (data not shown).

No changes in bone-turnover, measured by calcein double labeling, were present in SCD mice compared to controls, which was combined with similar levels of carboxy-terminal collagen crosslinks (CTX) in both mouse strains (data not shown).

These data suggest that SBD is sustained by a shift in the balance between osteoblast/osteoclast activity towards osteoclasts beside the up-regulation of Prx2 anti-oxidant system as back-up mechanism to control oxidative stress and limit osteoclast activity in SCD mice.

**Zoledronic acid prevents bone loss and ameliorates osteogenic differentiation in SCD mice**

Up-to-now, zoledronic acid is considered the most powerful amino-bisphosphonate that inhibits osteoclast activity and osteoclastogenesis and promotes osteogenic differentiation.\textsuperscript{27,28} We then treated SCD and control mice with zoledronic acid (Zol: 100 ug/Kg ip; single dose)\textsuperscript{23} to investigate whether it might effectively counteract bone impairment of SCD mice. Zol significantly reduced osteoclast number and erosion surface in SCD mice without major changes in healthy mice (Fig. 1E). This was associated with up-regulation of Runx2 and Sparc (Osteonectin) and down regulation of Rank genes expression in both mouse models (Fig. 2S). Prx2 expression was markedly decreased in Zol treated SCD mice compared to vehicle treated ones (Fig. 2S).

Taken together, these data suggest that in SCD mice, Zol decreases osteoclastic activity and promotes osteoblastogenesis in SCD mice.
Hypoxia/reoxygenation stress mimics acute vaso-occlusive crisis and increases bone turnover in SCD mice

Previous studies have shown that hypoxia/reoxygenation (H/R) stress mimics acute vaso-occlusive crisis (VOC) in different mouse models for SCD. Thus, we exposed SCD and healthy mice to H/R stress. As previously reported, H/R stress induced increased neutrophil count and decreased Hb levels (Table 1S). Increased bone turnover induced by H/R stress was present in both mouse groups and was higher in SCD mice compared to controls (Fig. 2A; Table 2S). This was associated with significant increased in osteoclast number and erosion surface only in SCD mice (Fig. 2B). Bone ALP increased in both AA and SS mice exposed to H/R, while and CTX significantly increased only in SCD mice (Fig. 2C).

In both mouse strains, H/R stress induced a down-regulation of Runx2 and Sparc (Osteonectin), suggesting reduced osteoblast differentiation (Runx2) and activity (Sparc; Osteonectin) (Fig. 3S). This was associated with up-regulation of RankL and Rank, indicating increased osteoclast activity and recruitment (Fig. 3S).

Zol, administrated before or after H/R stress, prevented the H/R induced increased bone turnover in both mouse strains. It is of interest to note that in SCD mice, pre-H/R administration of Zol was more efficient in reducing bone turnover than Zol administrated after H/R stress (Fig. 2D). Zol treatment was also able to significantly reduce osteoclast number and activity in both mouse strains (Fig. 2E).

Since the administration of Zol before H/R event seemed to be more effective on bone homeostasis than after H/R, we analyzed molecular markers of osteoblast/osteoclastogenesis in mice without or with Zol treatment administrated before H/R event. Zol prevented the H/R induced down-regulation of Runx2 and Sparc and H/R induced up-regulation of RankL and Rank gene expression in both mouse strains (Fig. 3A). We then analyzed Il6 gene expression in mice exposed to H/R stress with and without Zol treatment. As shown in Fig. 3B, Zol prevented the H/R induced up-regulation of Il6 in both mouse strains, possibly through the modulation of H/R induced increased osteoclast activity as previously reported for other aminobisphosphonates. Consistently with reduction in osteoclast activity and recruitment, we observed a down-regulation of H/R induced increased Prx2 expression in both mouse strains (Fig. 3C). No further changes in bone microarchitecture related to a single H/R event was observed in both healthy and SCD mice (data not shown).

The present data suggest that each acute VOC promotes osteoclast recruitment.
and activation, increases bone turnover, inhibits osteogenesis and promotes bone loss in SCD mice. Zol efficiently prevents H/R induced bone impairment and may be considered an interesting new treatment to prevent or limit SBD.

**In SCD mice, recurrent H/R events cause severe bone impairment and bone loss**

Since SCD patients experience recurrent VOCs, we exposed SCD and healthy mice to recurrent H/R stress (10 hrs 8% oxygen followed by reoxygenation) for 3 times with 3 weeks interval. In SCD mice, the recurrent VOCs worsened anemia and was associated with a marked increased in neutrophil count compared to either normoxic SCD mice or healthy mice exposed to recurrent H/R stress (Table 3S). As shown in Fig. 4A and B, recurrent VOCs severely worsened bone microarchitecture as supported by increased marrow star volume, reduced node numbers and node/termini ratio compared to controls (right panel Fig. 4A, Fig. 4SA). SCD mice undergoing recurrent VOCs showed also reduced trabecular bone volume and number associated with increased trabecular separation compared to normal controls (Fig. 4B). We did not observed major differences in osteoblasts numbers and osteoid surface between the two mouse strains, suggesting that recurrent H/R events affect also bone homeostasis in control mice (data not shown). Osteoclast number and erosion were significantly higher in SCD mice compared to controls (Fig. 4C). It is of interest to note that in health and SCD mice exposed to recurrent H/R events, the osteoclasts number and erosion were significantly higher than those observed under normoxic condition in both mouse strains ($P<0.02$ normoxia vs H/R, Fig. 1C, Fig. 4C).

In recurrent H/R exposed mice, calcein double labeling revealed a higher bone turnover in SCD compared to healthy mice (Fig. 5A; BFR/BS and AcF). In addition, this was higher in both mouse strains when compared to bone turnover under normoxia ($P<0.02$ normoxia vs H/R, Fig. 5A, Fig. 1C).

These data indicate that in SCD mice, the recurrent H/R events result in bone loss with severe alteration of bone structure and increased bone turnover. Consistently, *Runx2* was down-regulated; whereas, *RankL*, *Rank* and *Cathepsin k* were up-regulated in SCD mice compared to healthy mice (Fig. 5B). *Il6* and *Prx2* expression were again higher in SCD mice than in controls, supporting the contribution of oxidative stress in SBD (Fig. 4SB).

**Zoledronic acid restrains osteoclast activity, ameliorates bone microarchitecture**
and prevents bone loss in SCD mice exposed to recurrent H/R events

In order to evaluate the impact of zoledronic acid on SBD resulted from recurrent H/R events, we treated SCD and control mice with Zol (100 ug/Kg ip; single dose) one month before the first of the 3 repeated VOC events. As shown in Fig. 4A, Zol significantly prevented the H/R induced microarchitecture alterations, maintained bone volume and trabecular thickness, indicating that Zol prevents bone loss and ameliorates bone microarchitecture in SCD mice (Fig. 4A, B). These findings agreed with the reduction in osteoclast number and erosion surface in Zol treated SCD mice compared to vehicle treated ones (Fig. 4C). No major differences were observed in osteoblast number in both Zol and vehicle treated mice (data not shown). The recurrent H/R stress induced bone turnover was significantly decreased in both Zol treated SCD and AA mice compared to vehicle treated mice (Fig. 5A). In addition, Zol treatment significantly increased osteoblastogenesis (Runx2) and decreased osteoclast activity (RankL, Cathepsin k) and recruitment (Rank) in both mouse strains exposed to recurrent H/R stress (Fig. 5B). This was associated with down-regulation of Il6 and Prx2 (Fig.4SB) expression in Zol treated SCD mice compared to vehicle treated ones, in agreement with the reduction of osteoclast activity.

Our results indicate that Zol prevents the development of SBD in SCD mice exposed to recurrent VOCs.

DISCUSSION

SBD greatly impacts SCD patient quality of life and its clinical management is still unsatisfactory. Limited studies on SBD are available and its pathogenesis has been only partially investigated. 18,43-47

Here, we show bone impairment and bone loss in SCD mice under normoxic condition. This was associated with down-regulation of osteogenic markers (Runx2 and Sparc) and increased osteoclast activity (RankL) without major changes in osteoclastogenesis. Since Runx2 and Sparc explore osteoblast recruitment and differentiation, our findings suggest a decrease mesenchymal stem cell (MSCs) commitment in osteogenic lineage in SCD mice. Indeed, we observed a significant reduction in the number of CFU-Ob from SCD mice compared to healthy controls (Fig. 1E). supporting the perturbation of osteoprogenitor compartment in favor of osteoclast activity in SCD mice.

Based on these evidences, we chose zoledronic acid as powerful inhibitor of
osteoclast activity and promoter of osteogenic differentiation to treat SBD.\textsuperscript{27,28} In normoxic SCD mice, zoledronic acid reduced osteoclast activity and improved osteoblastogenesis as supported respectively by the down-regulation of \textit{Rank} and the up-regulation of \textit{Runx2} and \textit{Sparc} (Fig. 2S).

Acute vaso-occlusive events play a key role in the pathogenesis of sickle cell related organ damage and may contribute to SBD.\textsuperscript{12} In this study, we demonstrated that H/R stress, mimicking acute VOCs, increases bone turnover, further promotes osteoclast activity (\textit{RankL}) and osteoclastogenesis (\textit{Rank}) and further suppresses osteogenic lineage (\textit{Runx2}, \textit{Sparc}) (Fig. 2, 3S). In SCD mice, zoledronic acid markedly prevented the H/R induced increased bone turnover (Fig. 2), osteoclast activity (\textit{RankL}) and osteoclast recruitment (\textit{Rank}) in favor of osteoblastogenesis (\textit{Runx2}, \textit{Sparc}) (Fig. 3). This was associated with a down-regulation of H/R induced increased gene expression of \textit{Il6} and \textit{Prx2}, in agreement with the reduction in osteoclast activity (Fig. 3B, C). Similar effects of H/R stress and zoledronic acid treatment were also observed in healthy mice, but to a lower extent compared to SCD ones. Our data strongly indicate the key role of H/R stress in development of SBD. This is also supported by the beneficial effects of zoledronic acid, which switchs-off osteoclast activity and osteoclastogenesis in favor of osteoblastogenesis, limiting bone impairment related to H/R stress. It is of interest to note that the positive effects of zoledronic acid on bone homeostasis seem to be more pronounced in SCD mice treated before the exposure to H/R stress than after H/R event.

Since SCD patients experience recurrent VOCs, we exposed SCD mice to repeated H/R stresses. We found that recurrent VOCs severely affected bone structure and increased bone turnover in SCD mice compared to either SCD normoxic group or to SCD mice exposed to a single VOC (Fig. 2, 4, 5). This was associated with up-regulation of \textit{RankL}, \textit{Rank}, \textit{Cathepsin k}, \textit{Il6}, as pro-resorptive cytokine, and down-regulation of \textit{Runx2}, indicating increased osteoclast recruitment and activity combined with suppression of osteoblastogenesis (Fig. 5). A single dose of zoledronic acid, one month before the repeated VOCs, prevented bone impairment and bone loss, promoting osteogenic lineage in SCD mice compared to vehicle treated SCD group (Fig. 4, 5).

Based on these evidences, we believe that osteoblasts are the sleeping beauty in the pathogenesis of SBD (Fig. 6). Thus, the therapeutic inhibition of osteoclast activity/osteoclastogenesis might only be partially effective in long-term management of SBD in the absence of stimulating osteogenic lineage treatment, which might be used as sequential therapeutic strategy after inhibitors of osteoclast activity. Data from SCD mice
treated with zoledronic acid, which is a potent inhibitor of osteoclast activity with a previously reported action also on osteoblast recruitment,\textsuperscript{48} support this working model (Fig. 6).

In conclusion, our findings support the view that SBD is related to osteoblast impairment, resulting from reduced osteoprogenitors and osteoblast activity, combined with increased osteoclast activity (Fig. 6). Recurrent VOCs, associated with local oxidative stress and release of pro—resorptive cytokine, such as IL6, further increase osteoclast activity and osteoclast recruitment associated with depression of osteoblastogenesis, generating a severe bone loss and bone impairment compared to either SCD normoxic or single H/R stress SCD mice (Fig. 6). Zoledronic acid might act on both osteoclast and osteoblast compartment as multimodal therapy to prevent SBD.

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CONFLICT OF INTEREST\textsuperscript{a} The Authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS
LDF and LDC designed the experiments, analyzed data and wrote the manuscript, MVT carried out molecular analysis, analyzed data and wrote the manuscript, AM carried out the experiments and analyzed the data; LP carried out the analysis of iron bone content; AnM carried out ELISA experiments and performed bone samples preparation; AS carried out animal experiments; VS and GZ carried out H/R experiments.

REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Sickle cell mice display bone impairment, increased osteoclast activity and suppression of osteoblastogenesis. Beneficial effects of zoledronic acid on osteoclast activity in sickle cell mice.

(A) Quantitative histomorphometry of the distal femur of healthy (AA) and sickle cell (SS) mice. **Upper panel.** Representative undecalcified section of distal femur stained with trichrome Goldner’s stain. **Lower panel.** Marrow*:V: Marrow star volume; NdN/TV: node number/Tissue Volume; NdN/Tm: node to termini ratio. Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice. (B) Bone Volume (BV)/ Tissue Volume (TV), trabecular thickness, trabecular number and trabecular separation measured by Bone Explora Nova 3.5 image analyzer. Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice. (C) ObS/ BS: Osteoblast Surface/Bone Surface; OS/BS: Osteoid Surface/Bone Surface; N.Oc/TA: Osteoclast Number/Tissue Area; ES/BS: Erosion Surface/Bone Surface. Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice. (D) Real-Time PCR analysis of *Runx2*, *Sparc*, *RankL*, *Rank*, *Il6*. Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice. (E) To assess the number of osteoprogenitors bone marrow derived cells were cultured in vitro under osteogenic differentiation conditions. The CFU-Ob obtained from MSCs of healthy (AA) and sickle cell
(SS) mice were stained by alizarin red (upper panel) and quantified (#CFU-Ob/dish) (lower panel); data are shown as means±SD (n=3; P<0.05 AA vs SS). (F) ES/BS: Erosion Surface/Bone Surface; N.Oc/TA: Osteoclast Number/Tissue Area in healthy (AA) and sickle cell (SS) mice with and without zoledronic acid treatment (100 ug/Kg ip). Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice; °P<0.05 compared to vehicle treated mice.

**Fig. 2. Hypoxia/reoxygenation stress (H/R) affects bone homeostasis in sickle cell mice. Zoledronic acid prevents H/R induced increased turn-over and osteoclast activation.** (A) **Left panel.** Representative images of calcein-labeled bone surface. **Right panel.** Bone formation rate (BFR/BS) and Activation Frequency (AcF) in healthy (AA) and sickle cell (SS) mice under normoxia and exposed to H/R stress. Data are shown as means±SD (n=6); *P<0.05 compared to AA; °P<0.05 compared to normoxic mice. (B) N.Oc/TA: Osteoclast Number/Tissue Area; ES/BS: Erosion Surface/Bone Surface in healthy (AA) and sickle cell (SS) mice under normoxia and exposed to H/R stress. Data are shown as means±SD (n=6); *P<0.05 compared to AA; °P<0.05 compared to normoxic mice. (C) Serum levels of bone alcaline phosphatase (bALP) and carboxy-terminal collagen crosslinks (CTX) in AA and SS mice under normoxia and exposed to H/R stress. Data are shown as means±SD (n=6); *P<0.05 compared to AA; °P<0.05 compared to normoxic mice. (D) **Left panel.** Representative images of calcein-labeled bone surface. **Right panel.** BRF/BS and AcF in AA and SS mice exposed to H/R stress without and with zoledronic acid treatment before (pre-Zol) or after (Zol-post) H/R stress. Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice; °P<0.05 compared to vehicle treated mice; °P<0.05 Zol-pre compared to Zol-post. (E) N.Oc/TA and ES/BS in AA and SS mice exposed to H/R stress without and with zoledronic acid treatment before (pre-Zol) or after (Zol-post) H/R stress. Data are shown as means±SD (n=6); *P<0.05 compared to AA; °P<0.05 compared to vehicle treated mice.

**Fig. 3. Hypoxia/reoxygenation stress (H/R) down-regulates osteobastogenesis, induces osteoclastogenesis, up-regulates peroxiredoxin-2, anti-oxidant system.** Zoledronic acid plays a preventing role in H/R induce molecular bone expression. *Runx2, Sparc, RankL, Rank (A), Il6 (B) and Prx2 (C)* mRNA fold change in healthy (AA) and sickle cell (SS) mice exposed to H/R stress without and with zoledronic acid treatment before (pre-Zol). The fold change for each gene expression was obtained by using two
different housekeeping genes (Gapdh and β-Actin). Data are shown as means±SD (n=6); *P<0.05; **P<0.005.

**Fig. 4. Recurrent hypoxia/reoxygenation stress H/R stress mimicking acute vaso-occlusive crisis (VOCs) worsens bone structure in sickle cell mice.** (A) Quantitative histomorphometry of the distal femur of healthy (AA) and sickle cell (SS) mice exposed to recurrent (Rec) acute vaso-occlusive crisis (VOCs) without and with zoledronic acid treatment (Zol). Left panel. Representative undecalcified section of distal femur stained with trichrome Goldner’s stain. Right panel. marrow*V (Marrow star volume); NdN/TV: node number/Tissue Volume; NdN/Tm: node to termini ratio. Data are shown as means±SD (n=6); P<0.05 compared to healthy mice; °P<0.05 compared to vehicle treated mice. (B) Bone Volume (V)/ Tissue Volume (TV), trabecular thickness, trabecular number and trabecular in healthy (AA) and sickle cell (SS) mice exposed to recurrent (Rec) acute vaso-occlusive crisis (VOCs) without and with zoledronic acid treatment (Zol). (C) N.Oc/TA: Osteoclast Number/Tissue Area; ES/BS: Erosion Surface/Bone Surface in healthy (AA) and sickle cell (SS) mice exposed to recurrent (Rec) acute vaso-occlusive crisis (VOCs) without and with zoledronic acid treatment (Zol). Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice; °P<0.05 compared to vehicle treated mice.

**Fig. 5. Recurrent acute vaso-occlusive crisis (VOCs) increases bone turn-over, down-regulates osteoblastogenesis and up-regulates osteoclastogenesis in sickle cell mice. Zoledronic acid prevents bone impairment induced by repeated VOCs.** (A) Left panel. Representative images of calcein-labeled bone surface. Right panel. Bone Formation Rate (BFR/BS) and Activation Frequency (AcF) in healthy healthy (AA) and sickle cell (SS) mice exposed to recurrent (Rec) acute vaso-occlusive crisis (VOCs) without and with zoledronic acid treatment (Zol). Data are shown as means±SD (n=6); *P<0.05 compared to healthy mice; °P<0.05 compared to vehicle treated mice. (B) Runx2, RankL, Rank and Cathepsin k mRNA fold changes in healthy (AA) and sickle cell (SS) mice exposed to recurrent (Rec) acute vaso-occlusive crisis (VOCs) without and with zoledronic acid treatment (Zol). Two different housekeeping genes (Gapdh and β-Actin) were used to perform real time PCR analysis. Data are shown as means±SD (n=6); *P<0.05; **P<0.005.
Fig. 6. Schematic diagram of development of sickle cell bone disease (SBD) in mice. SCD mice display increased osteoclast activity and reduced osteoblastogenesis resulting in reduced osteoid formation compared to healthy mice. H/R stress depresses osteoblastogenesis, increases osteoclast activity and osteoclastogenesis, promoting bone impairment. Recurrent H/R stresses further worse the unbalance between osteoblastogenesis and osteoclastogenesis/osteoclast activity resulting in bone loss and severe bone impairment. Zoledronic acid blocks osteoclast activity and osteoclastogenesis and is associated with increased osteoblast recruitment and osteoblastogenesis, preventing the development of SBD. Healthy mice (AA) and sickle cell disease (SS). H/R: hypoxia reoxygenation stress; MSCs: mesenchymal stem cells.
Fig. 2

A  Normoxia  H/R
AA  AA  AA

SS  SS  SS

BFR/BS (ratio %)

AcF (# cycle/y)

ES/BS (%)

N.Oc/TA (#/mm²)

bALP (mg/mL)

CTX (ng/mL)

D  H/R  H/R Zol-pre  H/R Zol-post
AA  AA  AA

SS  SS  SS

E  H/R  H/R Zol-pre  H/R Zol-post

BFR/BS (ratio %)

AcF (# cycle/y)

ES/BS (%)

N.Oc/TA (#/mm²)

bALP (mg/mL)

CTX (ng/mL)
Hypoxia-reperfusion affects osteogenic lineage and promotes sickle cell bone disease

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