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Effects of CB2 and TRPV1 Stimulation on Osteoclast Overactivity Induced by Iron in Pediatric Inflammatory Bowel Disease

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Background: The reduction of bone mineral density and osteoporosis have high impacts on the health of patients with inflammatory bowel diseases (IBD). We have previously shown that a dysregulated iron metabolism occurs in IBD and leads to a decrease in circulating iron concentration and excessive intracellular sequestration of iron. Studies suggest that iron overload significantly affects the bone, accelerating osteoclast (OC) differentiation and activation, promoting bone resorption. Moreover, we demonstrated that iron overload causes OC overactivity. The cannabinoid receptor type 2 (CB2) and the transient receptor potential vanilloid type-1 (TRPV1) are potential therapeutic targets for bone diseases. The aim of this study was to evaluate the roles of CB2 and TRPV1 receptors and of iron in the development of osteoporosis in pediatric IBD.

Methods: We differentiated OCs from peripheral blood mononuclear cells of patients with IBD and healthy donors and evaluated CB2 and TRPV1 receptor expression; OC activity, and iron metabolism by Western blot, TRAP assays, bone resorption assays, and iron assays. Moreover, we analyzed the effects of the pharmacological modulation of CB2 and TRPV1 receptors on OC activity and on the iron metabolism.

Results: We confirmed the well-known roles of CB2 and TRPV1 receptors in bone metabolism and suggested that their stimulation can reduce the OC overactivity induced by iron, providing new insights into the pathogenesis of pediatric IBD-related bone resorption.

Conclusions: Stimulation of CB2 and TRPV1 could reduce IBD-related osteoporosis due to their direct effects on OC activity and to modulating the iron metabolism.

Lay Summary

In this study, we provide new insights into the pathogenesis of inflammatory bowel disease (IBD)–related bone resorption, suggesting a role for iron. Cannabinoid receptor type 2 and transient receptor potential vanilloid type-1 stimulation could reduce IBD-related osteoporosis, directly affecting osteoclast activity and modulating iron metabolism.

Key Words: inflammatory bowel disease, osteoporosis, osteoclasts, iron metabolism, CB2, TRPV1

Introduction

Inflammatory bowel diseases (IBD) include Crohn's disease (CD) and ulcerative colitis (UC), which are chronic, relapsing, inflammatory disorders of the gastrointestinal tract often diagnosed in childhood.[1,2] Altered cytokine production has a pivotal role in the pathogenesis of IBD, causing an inflammatory state both at local and systemic levels.[3,4] In 10% to 40% of cases, patients with IBD present extraintestinal manifestations,[5,6] among which the most frequent are those affecting the bone tissue, such as osteoporosis (OP).[7,8] Bone mass is mostly acquired during childhood, and the early onset of IBD may compromise the final bone mineral density, affecting growth.[9,10] The primary mechanism in the pathogenesis of OP in patients with IBD is chronic inflammation. Proinflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1, IL-6, IL-7, and IL-17, increase the ratio of receptor activator of nuclear factor-κB ligand (RANK-L)/Osteoprotegerin (OPG), which promotes bone resorption.[11] Furthermore, chronic malabsorption and glucocorticoid therapy also play critical roles in the development of OP in IBD.[12] We have previously shown that a dysregulated iron metabolism occurs in IBD and leads to a decrease in circulating iron concentration and an excessive intracellular sequestration of iron.[13] The main factor responsible for this altered iron metabolism is hepcidin, which operates by binding to the iron transporter ferroportin 1 (FPN-1), causing its internalization and degradation.[14–17] The cytokine IL-6 is the most important mediator of hepcidin upregulation.[18] Several studies have demonstrated the IL-6-dependent upregulation of hepcidin in mouse models of IBD,[18,19] as well as the correlation between serum hepcidin levels and IL-6 concentrations.[13,19,20] Further

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evidence suggests that iron overload significantly affects the bone by accelerating osteoclast (OC) differentiation and activation, promoting bone resorption.[21,22] Interestingly, we demonstrated that iron overload, through the upregulation of the OC marker tartrate-resistant acid phosphatase (TRAP), causes OC overactivity.[23] Moreover, in a previous study we showed that a common cannabinoid receptor type 2 (CB2) variant, Q63R, which causes reduced CB2 function, is associated with several autoimmune and inflammatory diseases, such as IBD, suggesting that the receptor could be a possible target for suppressing proinflammatory and immune diseases.[24–27] Indeed, CB2, together with transient receptor potential vanilloid type-1 (TRPV1), is a potential therapeutic target for bone diseases.[28,29] While TRPV1 receptors play a role in stimulating OC activity, CB2 activation is inhibitory.[30,31] Stimulation of CB2 reverts the pro-osteoporotic effects induced by estrogen withdrawal in menopause[29,32] and iron overload in thalassemia major.[23] Moreover, recently, we suggested CB2 as a molecular marker to reduce bone mass loss in celiac disease.[33] Therefore, given our current understanding of these processes, the aim of this study was to evaluate the roles of CB2 and TRPV1 receptors and of iron in the development of OP in pediatric IBD. Moreover, we analyzed the effects of the pharmacological modulation of CB2 and TRPV1 receptors on OC activity and on the iron metabolism, using cultures of OCs derived from peripheral blood cells of patients with IBD.

Methods

Patients

Our study population included a total of 21 pediatric subjects aged less than 18 years, stratified into 3 groups on the basis of their clinical diagnosis: 7 were affected by UC (12 ± 2.8 years), 7 were affected by CD (10 ± 3.2 years), and 7 were non-IBD controls (CTR; 10 ± 4 years). The subjects presented to the Department of Woman, Child and General and Specialist Surgery at Vanvitelli University between September 2020 and April 2021. The CTR group consisted of subjects who underwent an oesophagogastroscopy or ileo-colonoscopy to either exclude an organic disease or to practice polypectomy, and who did not present any signs of mucosal inflammation or disease, except for 2 patients that had single and isolated juvenile polyps. The remaining patients had a final diagnosis of functional dyspepsia ($n = 2$) and of functional constipation ($n = 3$). All patients with IBD were enrolled at diagnosis, before the start of medical treatment. The diagnoses of UC and CD were based on clinical, endoscopic, radiologic, and histopathologic criteria.[34] We collected 3 ml of blood from each enrolled subject. All procedures performed in this study were in accordance with the Helsinki Declaration of Principles, the Italian National Legislation, and the Ethics Committee of the University of Campania Luigi Vanvitelli (identification code Prot. 0013347/i), which formally approved the study.

OC Cell Cultures

Primary cultures of OCs were differentiated from the peripheral blood mononucleated cells (PBMCs) as previously described.[33] The PBMCs were isolated by centrifugation over Histopaque 1077 density gradient (Sigma Chemical), diluted at 1×10^6 cells/mL in α-minimal essential medium (Gibco), and supplemented with 10% fetal bovine serum (Euroclone), 100 IU/mL of penicillin, 100 g/mL of streptomycin (Gibco), and L-glutamine. The PBMCs were cultured in 24-well plates for 21 days in the presence of 25 ng/mL recombinant human macrophage colony-stimulating factor (Peprotech) and 50 ng/mL RANK-L (Peprotech) in order to have differentiated OCs. The culture medium was changed every 3 days with fresh medium.

Drugs and Treatments

Osteoclasts were treated with JWH-133 (a potent CB2 selective agonist) and resiniferatoxin (RTX; a potent analog of capsaicin that is an agonist at vanilloid receptors). The JWH-133 and RTX (Tocris) were dissolved in phosphate-buffered saline containing dimethyl sulfoxide (DMSO). The DMSO final concentration on cultures was 0.01%. Osteoclasts were treated with JWH-133 (100 nM) and RTX (5 μM) for 48 hours. Nontreated, cultured cells were preserved in incubation media during the same treatment time, with or without vehicle (DMSO 0.01%). The concentrations of drugs were determined through concentration-response experiments and were those inducing the strongest effect without altering cell viability.

Protein Isolation and Western Blot

Proteins were isolated from OC cultures through radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Millipore), according to the manufacturer's instructions. The Bradford dye-binding method (Bio-Rad) was used to quantify the total protein concentrations. The expression of CB2, TRPV1, TRAP, cathepsin K, FPN-1, and divalent metal transporter-1 (DMT1) in total lysates from OC cultures was determined by Western blot. Fifteen micrograms of denatured protein were loaded. Membranes were incubated with rabbit polyclonal anti-CB2 (1:500 dilution; Elabscience), rabbit polyclonal anti-TRPV1 (1:1000 dilution; Abcam), mouse monoclonal anti-TRAP (1:200 dilution; Santa Cruz), rabbit monoclonal anti–cathepsin K (1:1000 dilution; Abcam), rabbit polyclonal anti-FPN-1 (1:1000 dilution; Novus), mouse monoclonal anti-DMT1 (1:100 dilution; Santa Cruz) overnight at 4 °C and then with the specific secondary antibody for 1 hour. Chemiluminescence was used to reveal reactive bands (Immobilion Western Millipore) on a C-DiGit blot scanner (LI-COR Biosciences). A mouse monoclonal anti-β-actin antibody (1:100 dilution; Santa Cruz) was used as a housekeeping protein to verify the protein loading. Images were captured and analyzed using the Image Studio Digits software, version 5.0.

TRAP Assay

Tartrate-resistant acid phosphatase was evaluated as a specific OC marker and quantified through the acid phosphatase (ACP) method (Takara Bio), as previously described.[33] Cells were first fixed for 5 minutes at room temperature using a citrate buffer with a pH of 5.4 containing 60% acetone and 10% methanol. Then 50 μL of chromogen substrate solution (naphtol-AS-BI-phosphate substrate/fast red violet LB), mixed with 0.1 volume of sodium tartrate, was added to each well. The TRAP enzyme, cleaving the substrate, produces a red azoic dye with a purplish red color that can be revealed with an optical microscope (Nikon Eclipse TS100, Nikon Instruments). The TRAP(+) and multinucleated-OCs (more than 3 nuclei) were counted in at least 3 different wells in

each group of patients and treatment. To guarantee the functionality of the assay in each experiment, a positive and a negative control were included.

Bone Resorption Assay

The bone resorption assay was performed through a commercially available kit (CosMo Bio), as previously described.[33] Osteoclasts were differentiated from PBMC in a calcium phosphate–coated 24 multiwell. Receptor activator of nuclear factor-κB ligand was used at 100 ng/ml. At day 12, JWH-133 (100 nM) and RTX (5 μM) were added for 48 hours. At day 14, cells were removed using 5% sodium hypochlorite to observe and count the reabsorption pits with an optical microscope (Nikon Eclipse TS100, Nikon Instruments).

Iron Assay

After a 48-hour treatment, cell culture supernatants were collected to measure iron (III) using the Iron Assay Kit (Abcam), following the manufacturer's instructions. Standard and OC supernatants were pipetted into the wells and were incubated with an acidic buffer to consent iron release. Then, an iron probe was added at 25 °C for 60 minutes, in the dark. Released iron reacted with the chromogen, resulting in a colorimetric (593 nm) product, proportional to the iron content. A spectrophotometer (Tecan Infinite M200, Tecan Group Ltd.) was used to measure the optical density at a wavelength of 593 nm. Iron (II) and total iron (II + III) contents of the test samples (nmol/μL) were determined against a standard concentration curve. The iron (III) content can be calculated as: iron (III) = total iron (II + III) – iron (II).

Statistical Analysis

Results are expressed as means ± standard deviations (SDs). The experiments (TRAP assay and bone resorption assay) on cells were conducted in triplicate on each individual sample. Biochemical data (Western blotting and iron assay) were

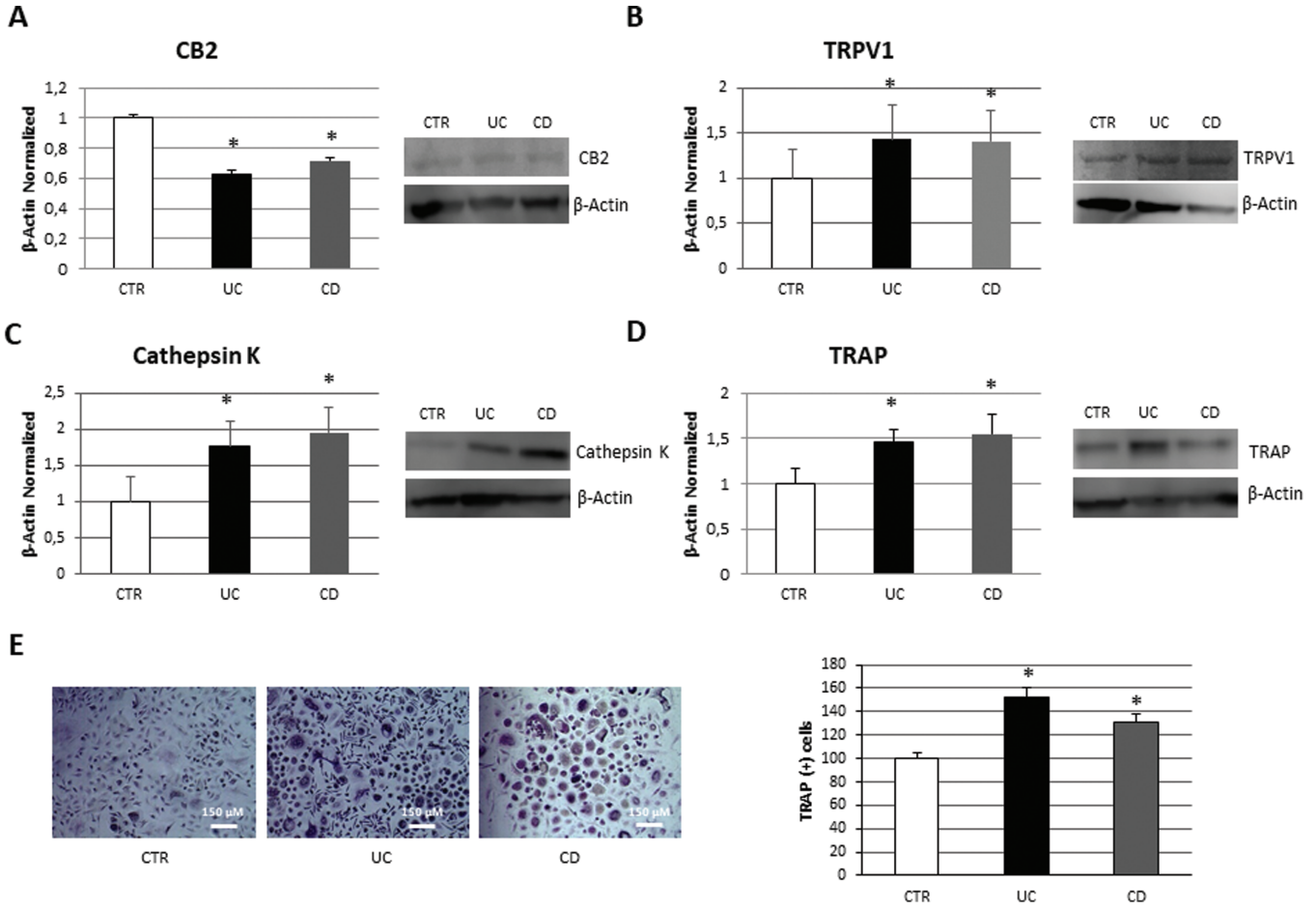

Figure 1. (A) CB2, (B) TRPV1, (C) cathepsin K, and (D) TRAP protein expression in OCs from 7 patients with UC and 7 patients with CD compared with OCs from 7 healthy donors (CTR). Protein levels were determined by Western blot, loading 15 μg of total lysate. The most representative images are displayed. The protein bands were detected through Image Studio Digits software. The intensity ratios of immunoblots, compared to CTR, taken as 1, were quantified after normalizing with respective controls. The relative quantifications for CB2, TRPV1, cathepsin K, and TRAP expression, normalized for the housekeeping protein β-actin, are represented in histograms as the mean ± SD of independent experiments on each individual sample. (E) TRAP assay on 7 patients with UC and 7 patients with CD compared with OCs from 7 CTR. The most representative images are displayed. TRAP (+) multinucleated ($n \geq 3$) OCs, stained in purple, were counted through an AE2000 inverted microscope at 10x magnification, in at least 3 different wells for each individual sample. The percentage number of TRAP (+) cells respective to the total cell number for each sample is presented in the histogram as the mean ± SD. A Student *t*-test was used for the statistical analysis. *$P \leq .05$ compared to CTR. Abbreviations: CB2, cannabinoid receptor type 2; CD, Crohn's disease; CTR, controls; OC, osteoclasts; SD, standard deviation; TRAP, tartrate-resistant acid phosphatase; TRPV1, transient receptor potential vanilloid type-1; UC, ulcerative colitis.

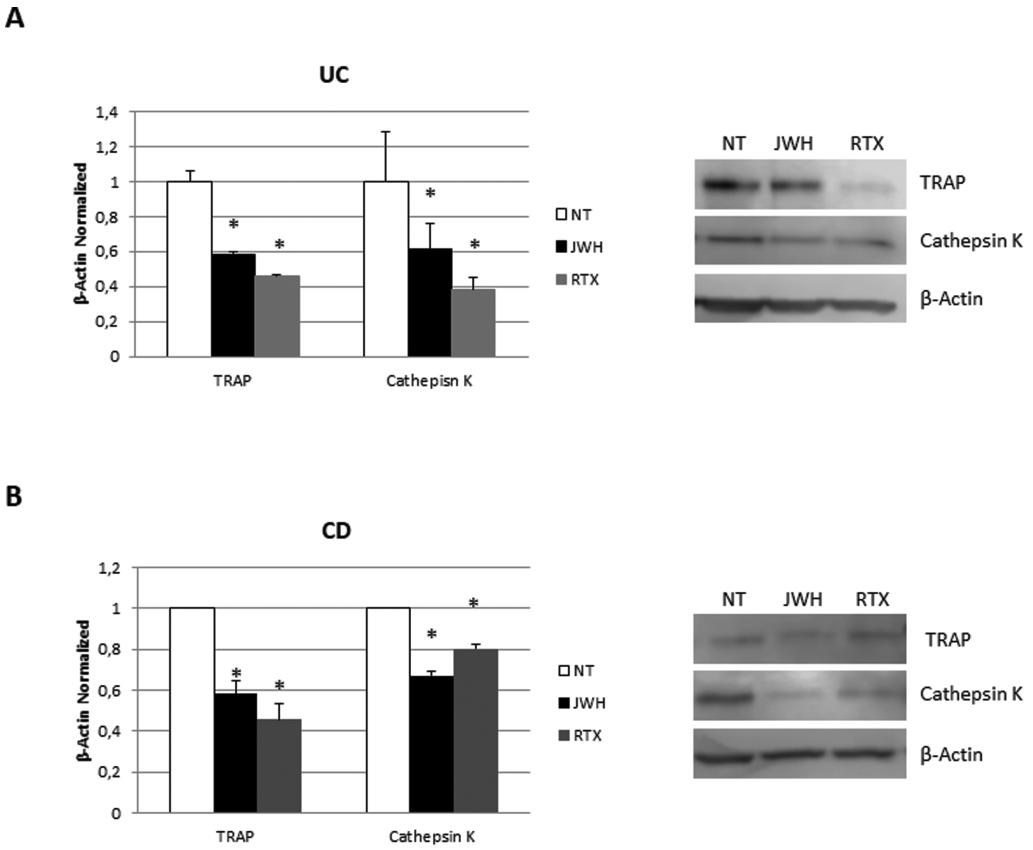

Figure 2. TRAP and cathepsin K protein expression in OCs from (A) 7 patients with UC and (B) 7 patients with CD after 48 hours of exposure with JWH-133 (100 nM) and RTX (5 μM). Protein levels were determined by Western blot, loading 15 μg of total lysates. The most representative images are displayed. The protein bands were detected through Image Studio Digits software. The intensity ratios of immunoblots compared to the NT, taken as 1, were quantified after normalizing with respective controls. The relative quantifications for TRAP and cathepsin K expression, normalized for the housekeeping protein β-actin, are presented in the histograms as the mean ± SD of independent experiments on each individual sample. A Student *t*-test was used for the statistical analysis. *$P \leq .05$ compared to the NT. Abbreviations: CD, Crohn's disease; NT, untreated control; OC, osteoclasts; RTX, Resiniferatoxin; SD, standard deviation; TRAP, tartrate-resistant acid phosphatase; UC, ulcerative colitis.

obtained from independent experiments on each individual sample. Statistical analyses on all data were performed using the Student *t*-test (XLSTAT by Addinsoft 2020) to evaluate differences between quantitative variables. Data are expressed as means ± SDs. A P value ≤ .05 was considered statistically significant.

Results

Characterization of OCs Derived From Patients With IBD

In order to characterize OCs derived from patients with IBD, we performed a Western blot to evaluate protein expression levels of CB2 and TRPV1 receptors and of 2 specific OC biomarkers, TRAP and cathepsin K (Figure 1). The expression of CB2 was significantly lower in OCs from patients with UC and CD than in OCs from healthy subjects (CTR; Figure 1A). Accordingly, TRPV1 was upregulated in both UC and CD OCs (Figure 1B). Thus, as previously demonstrated, decreased CB2 receptors with increased TRPV1 activity may explain the development of OP in patients with IBD. Moreover, as expected, both TRAP and cathepsin K were highly expressed in OCs from patients with UC and CD (Figure 1C and D). Tartrate-resistant acid phosphatase was also evaluated by performing a TRAP assay, a colorimetric assay that identifies TRAP (+) and multinucleated ($n \geq 3$) OCs. In accordance with biochemical data, the assay revealed increases in the numbers of TRAP (+) cells in patients with UC and CD. Interestingly, we observed increased sizes of OCs derived from patients with IBD (mean diameter: CTR, 70 μm; UC, 110 μm; CD, 130 μm; Figure 1E).

Effects of CB2 and TRPV1 Modulation on OC Activity and Numbers

To highlight the roles of CB2 and TRPV1 receptors in the pathogenesis of OP in IBD, we treated *in vitro* UC- and CD-derived OCs with the CB2 agonist JWH-133 (100 nM) and the TRPV1 agonist RTX (5 μM), evaluating the effects on OC activity. As revealed by Western blot, JWH-133 induced significant decreases of TRAP and cathepsin K expression in both groups of patients (Figure 2A and B). We also observed a reduction of OC biomarkers when OCs from patients with IBD were treated with the TRPV1 agonist RTX (5 μM), suggesting that the effect observed was due to the activation and subsequent desensitization of the channel, as previously demonstrated in other conditions characterized by the upregulation of TRPV1. The TRAP assay revealed a significant decrease in OC numbers in

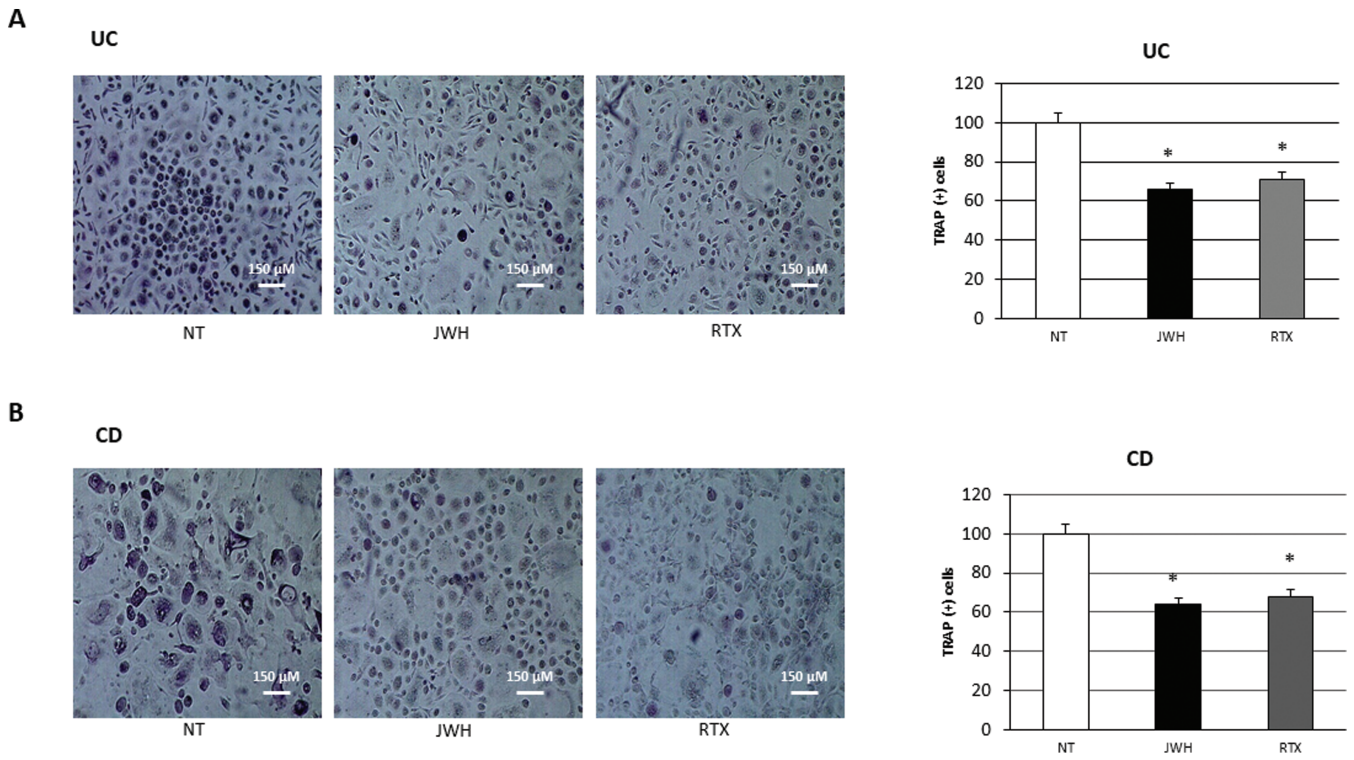

Figure 3. TRAP assays on (A) 7 patients with UC and (B) 7 patients with CD after 48 hours of exposure with JWH-133 (100 nM) and RTX (5 µM). The most representative images are displayed. TRAP (+) multinucleated ($n \geq 3$) OCs, stained in purple, were counted through an AE2000 inverted microscope at 10x magnification, in at least 3 different wells for each individual sample. The percentage number of TRAP (+) cells respective to the total cell number for each sample is presented in the histogram as the mean ± SD. A Student *t*-test was used for the statistical analysis. *$P \leq .05$ compared to NT. Abbreviations: CD, Crohn's disease; NT, untreated control; OC, osteoclasts; RTX, Resiniferatoxin; SD, standard deviation; TRAP, tartrate-resistant acid phosphatase; UC, ulcerative colitis.

UC- and CD-derived OCs after both CB2 and TRPV1 activation (Figure 3).

Effects of CB2 and TRPV1 Modulation on Bone Resorption Induced by RANK-L

To evaluate the effects of CB2 and TRPV1 modulation on RANK-L-induced bone resorption, we also performed a bone resorption assay, evaluating the area reabsorbed by OCs. We showed that the pharmacological stimulation of CB2 and TRPV1 with JWH-133 (100 nM) and RTX (5 µM), respectively, reduced the pits area, exerting a significant inhibitory effect on RANK-L-induced bone resorption (Figure 4).

Evaluation of the Iron Metabolism in OCs Derived From Patients With IBD

To evaluate the iron metabolism in OCs derived from patients with IBD, we measured the intracellular ferric iron ion concentration (Fe3+) and performed a Western blot to analyze the expression levels of 2 important regulators of iron metabolism: ferroportin, the only known iron exporter protein, and the iron transporter DMT1 (Figure 5). We observed significant increases of Fe3+ in OCs from patients with UC and CD compared to the CTR subjects, suggesting a possible key role of iron in the pathogenesis of OP in IBD (Figure 5A). Moreover, we observed a reduction of FPN-1 and an increase of DMT1, confirming the suggested hypothesis (Figure 5B and C).

Effects of CB2 and TRPV1 Modulation on the Iron Metabolism

To analyze the effects of CB2 and TRPV1 modulation on the iron metabolism in OCs derived from patients with IBD, we performed a Western blot to reveal ferroportin and DMT1 protein expression levels after 48 hours of exposure to JWH-133 (100 nM) and RTX (5 µM; Figure 6). We observed significant increases of ferroportin levels in OCs from patients with UC and CD after both the treatments (Figure 6A). Moreover, CB2 and TRPV1 modulation was also able to reduce DMT1 levels in a significant manner (Figure 6B).

Discussion

To the best of our knowledge, this manuscript is the first to present a framework for the analysis of the roles of CB2 and TRPV1 receptors in the development of OP in pediatric IBD. Consistent with previous findings, our data demonstrated the implication of the receptors in IBD OC activation. Osteoclasts from patients with UC and CD expressed higher levels of TRPV1 and lower levels of CB2 compared to OCs from healthy controls. In conjunction with the increased resorption activity in patients with IBD, we found significant upregulation of the OC markers TRAP and cathepsin K and, consequently, OC hyperactivation. Considering the potential anti-osteoporotic effects of CB2 stimulation and TRPV1 desensitization, we treated OCs

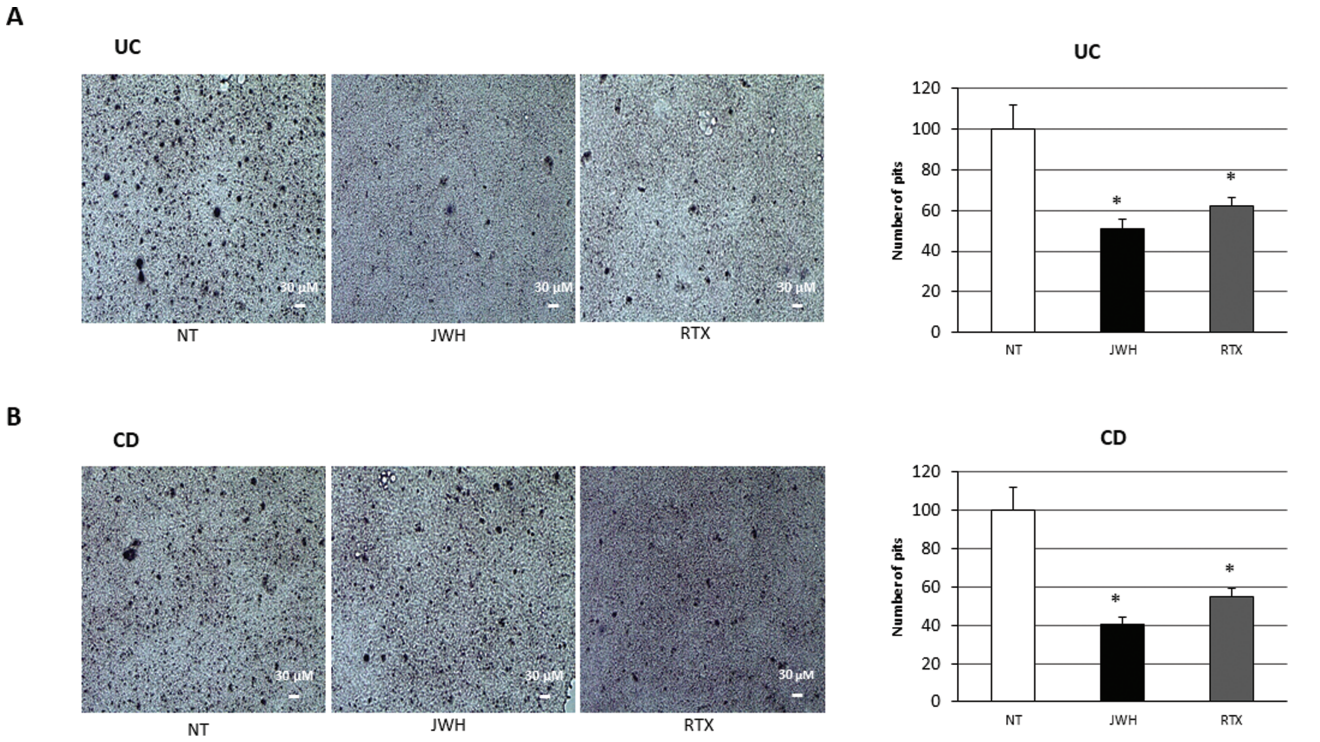

Figure 4. Bone resorption assays on (A) 3 patients with UC and (B) 3 patients with CD after 48 hours of exposure with JWH-133 (100 nM) and RTX (5 µM). The most representative images are displayed. The reabsorbed areas on the plate were visualized through an AE2000 inverted microscope at 10x magnification, in at least 3 different wells for each individual sample. The percentage numbers of the reabsorption pits (visualized in images as darker areas) are presented in histograms as the mean ± SD. A Student *t*-test was used for the statistical analysis. **P* ≤ .05 compared to NT. Abbreviations: CD, Crohn's disease; NT, untreated control; RTX, Resiniferatoxin; SD, standard deviation; UC, ulcerative colitis.

from patients with UC and CD with the CB2 agonist JWH-133 and the TRPV1 agonist RTX. Both the treatments led to reductions in osteoclastic hyperactivation, affecting bone marker expression, the number of OCs, and the bone resorption area. As previously observed in other pathological conditions in which TRPV1 is upregulated, RTX induces TRPV1 desensitization, thus exerting the same effect of TRPV1 receptor antagonism.[35] Therefore, our data suggest that pharmacological compounds stimulating CB2 or inhibiting or desensitizing TRPV1 could also be used as antiresorptive agents in IBD. The OC biomarker TRAP is an iron-phosphoesterase, and its activation is regulated by iron.[36] A dysregulated iron metabolism occurs in IBD and leads to a decrease in the circulating iron concentration and to excessive intracellular sequestration of iron.[13,37] Several studies suggest that iron overload significantly affects the bone by accelerating OC differentiation and activation, thus promoting bone resorption.[21] We previously demonstrated that iron overload causes OC overactivity, through TRAP upregulation.[23] Therefore, we analyzed the Fe3+ concentration and the expression of important modulators of iron metabolism in OCs derived from patients with IBD to demonstrate an increase of Fe3+ storage, suggesting the involvement of iron in the pathogenesis of IBD-related OP. The main factor responsible for the altered iron metabolism in IBD is hepcidin, whose secretion is induced by inflammatory cytokines (IL-1β and IL-6).[3,16–18,38] Hepcidin acts by binding to the only known iron exporter protein FPN-1, causing its internalization and degradation.[14,39] Accordingly, we observed a decrease of FPN-1 in both patients with CD and UC, as compared to controls. The increased iron content in bone could also be a consequence of the upregulation of DMT1, a proton-coupled metal-ion transport protein that carries iron from the extracellular to the cytoplasm[40] and is highly expressed by mature OCs.[41] Accordingly, the OCs derived from patients with UC and CD showed increases in DMT1 levels. Divalent metal transporter-1 is regulated at transcriptional[42] and post-transcriptional[43] levels. While DMT1 phosphorylation induces transport activity, DMT1 dephosphorylation is associated with a loss of iron uptake.[44] Seo et al.[44] demonstrated that the cannabinoid delta-9 tetrahydrocannabinol inhibits the DMT1 function in human embryonic kidney 293 (HEK293) T-cells by blocking DMT1 phosphorylation. Recently, Jia et al.[45] also demonstrated that CB2 activation mediated by JWH-133 inhibits DMT1 expression and iron influx. In line with these studies, we observed a significant reduction of DMT1 expression in OCs from patients with IBD, together with a strong increase of FPN-1, suggesting a role for CB2 in modulating the iron metabolism in iron-overloaded OCs. We observed the same trend after the stimulation of the Ca^{2+} permeable channel TRPV1, demonstrating for the first time, to the best of our knowledge, a role for the channel in modulating iron homeostasis through FPN-1 and DMT1 protein expression. This interesting finding is in agreement with several studies that demonstrated the role of calcium channels in iron uptake under iron-overloaded condition.[46–50] In 2019, with a randomized, placebo-controlled study, Khaled et al.[50] demonstrated that when Amlodipine, a well-known calcium blocker, is added to the standard chelation therapy it

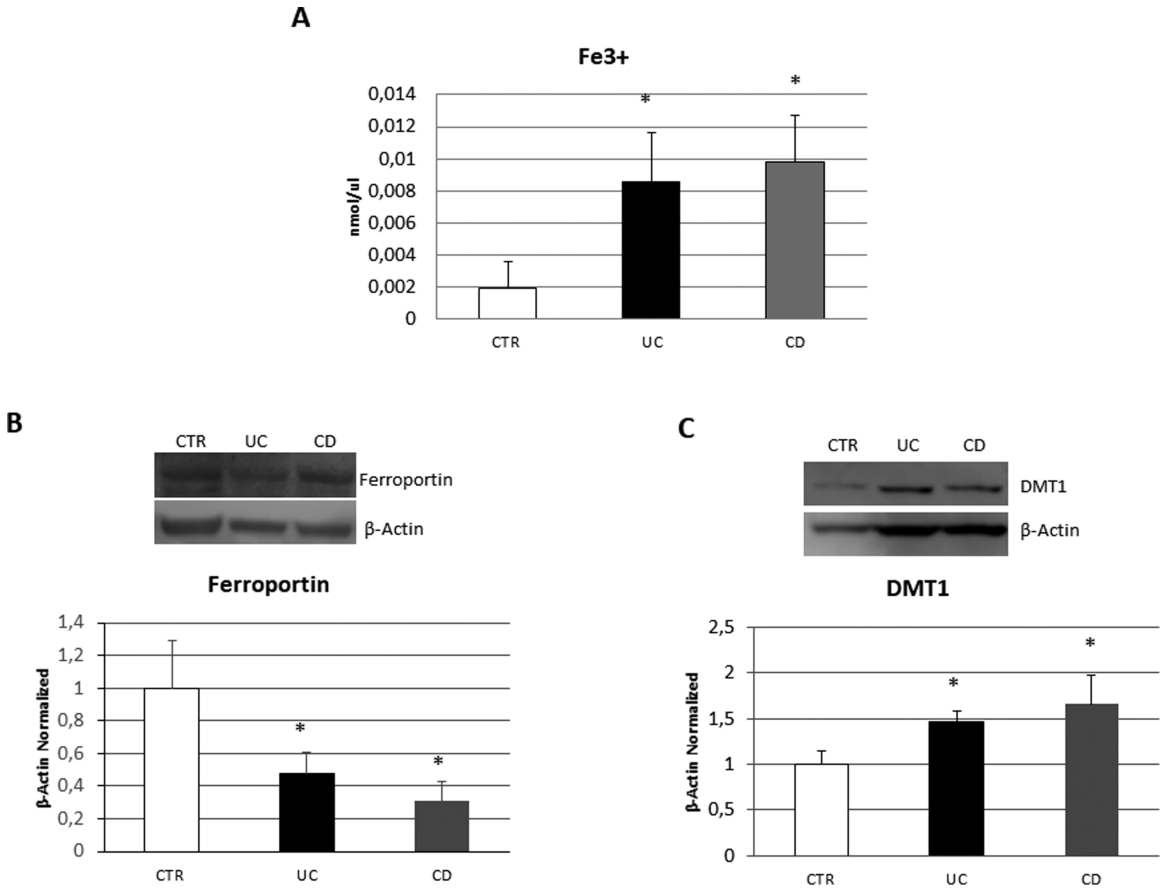

Figure 5. (A) Fe3+ intracellular concentrations (nmol/μL) in 7 patients with UC and 7 patients with CD compared with OCs from 7 healthy donors (CTR), determined by iron assay. The histogram shows the Fe3+ concentrations as the mean ± SD of independent experiments on each individual sample. (B) Ferroportin and (C) DMT1 protein expression in OCs from 7 patients with UC and 7 patients with CD compared with OCs from 7 healthy donors (CTR). Protein levels were determined by Western blot, loading 15 μg of total lysate. The most representative images are displayed. The protein bands were detected through Image Studio Digits software. The intensity ratios of immunoblots compared to CTR, taken as 1, were quantified after normalizing with respective controls. The relative quantifications for Ferroportin and DMT1 expression, normalized for the housekeeping protein β-actin, are represented in the histograms as the mean ± SD of independent experiments on each individual sample. A Student *t*-test was used for the statistical analysis. *$P \leq .05$ compared to CTR. Abbreviations: CD, Crohn's disease; CTR, controls; DMT1, divalent metal transporter-1; Fe3+, ferric iron ion concentration; OC, osteoclasts; SD, standard deviation; UC, ulcerative colitis.

reduces the iron concentration in patients with transfusion-dependent thalassemia major. Kumfu et al.[48] demonstrated that the L-type and T-type calcium channels are essential for Fe3+ uptake in cardiomyocytes under an iron-overloaded condition. In agreement, the T-type calcium channel blocker efonidipine improves the effects of the iron chelator Deferiprone in reducing cardiac iron depositions in iron-overloaded thalassemic mice.[49] Patients with IBD often experience nausea and a decreased appetite.[51] Although clinical studies are limited, cannabis has been shown to alleviate these symptoms in patients with IBD, improving their quality of life.[52–54] Chronic abdominal pain is among the most debilitating symptoms of IBD, and TRPV1 is known to modulate pain.[55] Recently, Defaye et al.[56] proved that the inhibition of the visceral TRPV1 prevents microglial activation in the spinal cord and, subsequently, prevents persistent visceral hypersensitivity in colitis mice, suggesting that the channel could induce the transition from acute to chronic pain during active remission. Therefore, the anti-osteoporotic effects induced by CB2 and TRPV1 stimulation, together with the already known effects of the receptors in reducing IBD symptoms, makes us confident that these compounds can be used in IBD. Certainly, a limit of our study is the *in vitro* treatment. It would be interesting to also confirm our data in the complex *in vivo* environment (microbiota, nutrition, and immune response) of the gut system. Notwithstanding the small sample size, the number of patients enrolled allowed us to obtain clear and reliable results, supported by a valid statistical analysis. Indeed, the possibility to obtain 21 samples from both healthy and IBD pediatric subjects at diagnosis is certainly an important and noteworthy strength of our study, both to enrich the number of samples of our study and to perform *in vivo* investigations. Despite these limitations, our data confirmed the well-known roles of CB2 and TRPV1 receptors in bone metabolism and suggested, for the first time, that iron has a crucial role in OC overactivity in pediatric IBD, providing new insights into the pathogenesis of IBD-related bone resorption. Stimulation of CB2 and TRPV1 could reduce IBD-related OP, with a direct effect on OC activity and a parallel, indirect effect modulating the iron metabolism.

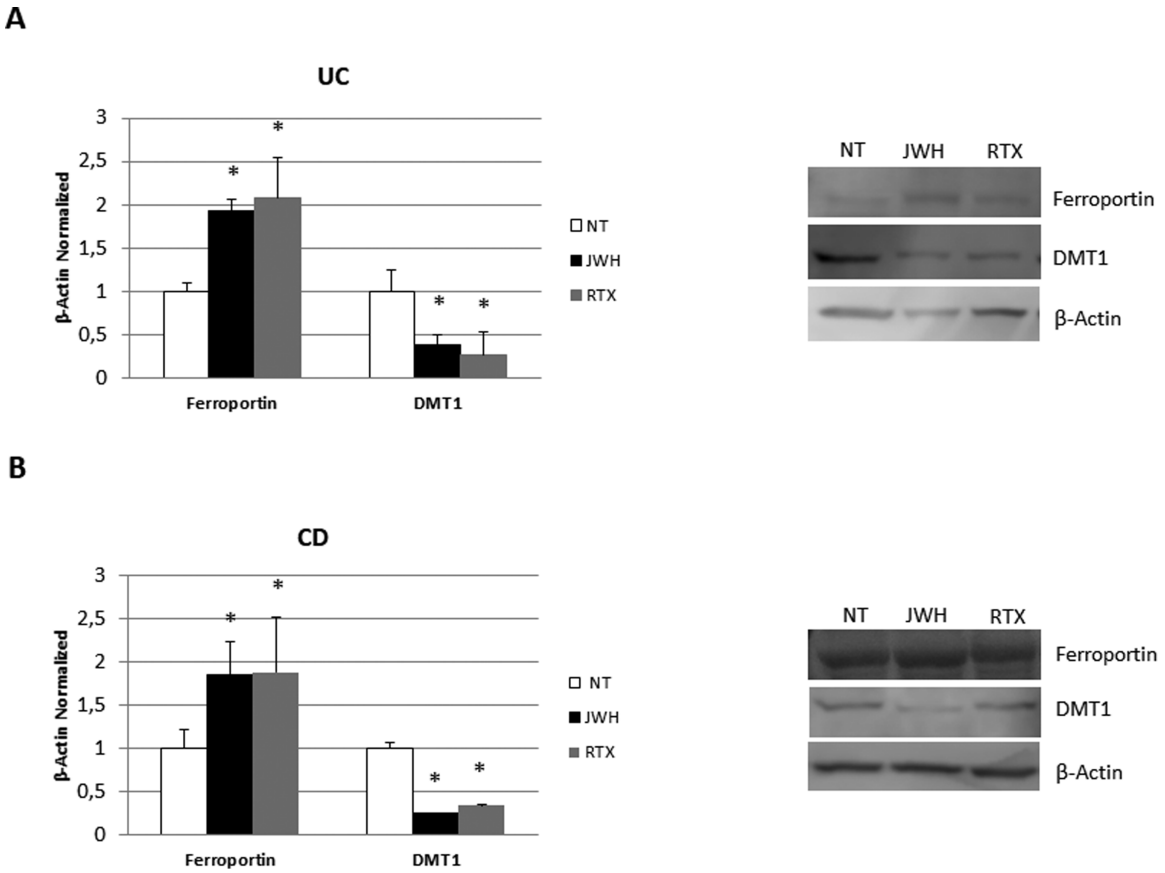

Figure 6. Ferroportin and DMT1 protein expression in OCs from (A) 7 patients with UC and (B) 7 patients with CD after 48 hours of exposure with JWH-133 (100 nM) and RTX (5 μM). Protein levels were determined by Western blot, loading 15 μg of total lysates. The most representative images are displayed. The protein bands were detected through Image Studio Digits software. The intensity ratios of immunoblots, compared to the NT, taken as 1, were quantified after normalizing with respective controls. The relative quantifications for Ferroportin and DMT1 expression, normalized for the housekeeping protein β-actin, are presented in the histograms as the mean ± SD of independent experiments on each individual sample. A Student *t*-test was used for statistical analysis. *$P \leq .05$ compared to the NT. Abbreviations: CD, Crohn's disease; DMT1, divalent metal transporter-1; NT, untreated control; OC, osteoclasts; RTX, Resiniferatoxin; SD, standard deviation; UC, ulcerative colitis.

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Author Contributions

C.T.: supervision, data curation, validation, and writing o-riginal draft preparation; A.D.P: methodology, data curation, formal analysis and validation. M.C.: data curation, formal analysis; M.A.: data curation, formal analysis, and validation; M.M.: investigation and resources; E.M.: investigation and resources; F.R.: project administration, funding acquisition, supervision, and writing original draft preparation; C.S.: conceptualization, project administration, funding acquisition, supervision, and writing original draft preparation

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Conflicts of Interest

None declared.

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