


RESEARCH ARTICLE

Evaluation of impaired growth plate development of long bones in skeletally immature mice by antirheumatic agents

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Abstract

Restriction of physical growth and development is a known problem in patients with juvenile idiopathic arthritis (JIA). However, the effect of medical treatment for JIA on skeletal growth in affected children has not been properly investigated. We, therefore, hypothesize that naproxen and methotrexate (MTX) affect endochondral ossification and will lead to reduced skeletal development. Treatment of ATDC5 cells, an in vitro model for endochondral ossification, with naproxen or MTX resulted in increased chondrogenic but decreased hypertrophic differentiation. In vivo, healthy growing C57BL/6 mice were treated with naproxen, MTX, or placebo for 10 weeks. At 15 weeks postnatal, both the length of the tibia and the length of the femur were significantly reduced in the naproxen- and MTX-treated mice compared to their controls. Growth plate analysis revealed a significantly thicker proliferative zone, while the hypertrophic zone was significantly thinner in both experimental groups compared to their controls, comparable to the in vitro results. Micro-computed tomography analysis of the subchondral bone region directly below the growth disc showed significantly altered bone microarchitecture in naproxen and MTX groups. In addition, the involvement of the PTHrP-Ihh loop in naproxen- and MTX-treated cells was shown. Overall, these results demonstrate that naproxen and MTX have a profound effect on endochondral ossification during growth plate development, abnormal subchondral bone morphology, and reduced bone length. A better understanding of how medication influences the development of the growth plate will improve understanding of endochondral ossification and reveal possibilities to improve the treatment of pediatric patients.

KEYWORDS

endochondral ossification, growth plate, JIA, MTX, naproxen

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1 | INTRODUCTION

The major part of the mammalian skeleton develops via a process in the growth plates named endochondral ossification.¹ Endochondral ossification of long bones typically starts with the formation of a cartilaginous tissue containing highly proliferative chondrocytes. This cartilaginous tissue then gradually transforms into hypertrophic and mineralized cartilage, followed by remodeling into bony tissue.¹ Growth plate activity is directly responsible for skeletal growth and its *de novo* bone-generating activity peaks during in utero development of the fetus, the first months after birth and during puberty.² During these phases of development, the growth plates are most vulnerable for disturbances that affect the endochondral ossification process and in turn may lead to abnormal skeletal development, leading to malformations, dwarfisms, gigantisms, and others.^{2,3}

Impairment of physical growth and development is seen in patients suffering from juvenile idiopathic arthritis (JIA).⁴ Thirty-nine percent of children with JIA experience delayed and restricted growth and have a reduced final body length compared to their peers,^{5,6} with significant implications for both physical and psychosocial health. Although initially reversible, long-standing growth plate impairment results in irreversible short stature (below the third percentile) and altered adult body stature.⁷ The growth impairment in JIA is often explained by the sustained systemic inflammation, reduced mobility, malnutrition, and catabolic imbalance in pediatric patients. However, the role of the currently used pharmacological treatment in the growth of the skeleton in JIA-affected children was only recently recognized.⁷

To manage inflammatory responses, reduce pain, and preserving joint function, JIA patients are pharmacologically treated for prolonged periods with nonsteroidal anti-inflammatory drugs (NSAIDs). Next to NSAID's JIA patients are treated with disease-modifying antirheumatic drugs; amongst others methotrexate (MTX). Classically, also glucocorticoids have been frequently used, but these have been shown to have an interfering action on growth, leading to a reduction in final adult height in these patients.⁸ The use of new antirheumatic biologicals such as etanercept and tocilizumab have been associated with impaired growth in JIA patients too.^{9,10} Despite the positive effects of MTX and NSAIDs, such as naproxen, on the inflammatory condition in JIA, the consequences of these drugs on endochondral ossification have not yet been sufficiently investigated.

The NSAID class of anti-inflammatory drugs mainly focuses on inhibiting cyclooxygenase (COX) activity and thereby reducing pain, inflammation, and fever. Impairment of growth plate development by impaired chondrocyte hypertrophy in the growth plate by selective COX-2 inhibition has been described before.¹¹ In spite of these observations, there is a controversy over the exact effects of COX inhibitors on the chondrogenic part of endochondral bone formation processes during skeletal growth itself.

MTX is used at high-doses for the treatment of malignancies¹² and at lower doses for the treatment of JIA. From studies in children affected with acute lymphoblastic leukemia (ALL), the bone metabolism after treatment with high dose MTX is disturbed, resulting in

reduced bone lengthening and bone loss. Based on these observations, pediatric patients suffering from JIA or ALL and receiving MTX over long periods could be at risk of suppression of skeletal growth.

Current treatment strategies of pediatric orthopedic or JIA patients commonly involve naproxen and MTX. To better understand how these mediations affect endochondral ossification and growth plate development, we sought to study the consequences of these two selected pharmacological agents in an *in vitro* model for endochondral ossification and in growth plate development of skeletally immature mice.

2 | MATERIALS AND METHODS

2.1 | ATDC5 experiment

ATDC5 cells (RIKEN BRC Cell Bank) were cultured in proliferation medium (Dulbecco's modified Eagle's medium/F12; Invitrogen, Carlsbad, CA), 5% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO), 1% antibiotic/antimycotic (Invitrogen), 1% nonessential amino acids (Invitrogen). Cells were plated at 6400 cells/cm². Chondrogenic differentiation was initiated by changing the medium to differentiation medium (proliferation medium supplemented with 10 µg/mL insulin [Sigma-Aldrich], 10 µg/mL transferrin [Roche, Basel, Switzerland], 30 nM sodium selenite [Sigma-Aldrich]) (day 0). Naproxen (final concentration of 1 mM; Sigma-Aldrich) or MTX (final concentration of 1 µM; Sigma-Aldrich) was added to the differentiation medium. Differentiation medium (with naproxen or MTX) was refreshed every 2 days. Samples were lysed in TRIzol (Life Technologies, Carlsbad, CA). RNA isolation from TRIzol, RNA quantification, complementary DNA synthesis, and real-time quantitative polymerase chain reaction were performed as described before.¹³ Validated primer sequences were used and presented in Table 1. Data were analyzed using the standard curve method, messenger RNA (mRNA) expression was normalized to the reference gene (β -actin) and gene expression was calculated as fold change as compared to day 0 conditions. In a parallel experiment, the glycosaminoglycan (GAG) content was measured using a standardized modified dimethyl methylene blue assay. GAG content was corrected for total protein content with a bicinchoninic acid assay (Sigma-Aldrich)

2.2 | Experimental design animal model

For this study 48 healthy skeletally immature (5 weeks old/ \pm 20 g) C57BL/6 mice were used (Charles River Laboratories, Wilmington, MA). The Maastricht University Animal Ethical Committee approved the experiment (MUMC DEC Approval 2014-084) and we confirm that all experiments were performed in accordance with relevant guidelines and regulations (ARRIVE). The sample size was calculated according to the formula of L. Sachs $n = (\sigma/\delta)^2 \times 15.7$, corrected for potential drop-out, and 12 animals per group were included (all females). Throughout the experiment, animals were

TABLE 1 Primer sequences for RT-qPCR

| Oligo sets mouse | Forward | Reverse |
|---------------------|-------------------------------|--------------------------|
| β-Actin | GACAGGATGCAGAAGGAGATTACTG | CCACCGATCCACACAGAGTACTT |
| Col2a1 | TGGGTGTTCTATTTATTTATTGTCTTCCT | GCGTTGGACTCACACCAGTTAGT |
| Sox9 | AGTACCCGCACCTGCACAAC | TACTTGTAGTCCGGGTGGTCTTTC |
| PTHrP | AAGGGCAAGTCCATCCAAGA | CTCGGCGGTGTGTGGATTTTC |
| Col10a1 | CATGCCTGATGGCTTCATAAA | AAGCAGACACGGGCATACCT |
| lhh | TGGACTCATTGCCTCCCAGA | CAAAGGCTCAGGAGGCTGGA |
| Runx2 | GACGAGGCAAGAGTTTCACC | GGACCGTCCACTGTCACTTT |
| Patched 1 | GCCTACAAACTCTGGTGCAA | TTTAGTCAACTGGCTGATGTCGAT |
| Patched 2 | GCTGGTCTGGCAATGATG | TCAGTTTGATGCCAGGAATC |
| Gli1 | GGACCTGCAGACGGTTATCC | GGAGATGTGCATCGCGAGTT |
| Gli3 | ACCATACGTCTGTGAGCACGAA | GCGCGATCAGAGGCATTT |

Note: The 5' to 3' forward and reverse oligonucleotide sequences used for RT-qPCR are listed in the table.

Abbreviation: RT-qPCR, real-time quantitative polymerase chain reaction.

housed in groups under standard conditions with ad libitum access to water and food and 12 hours of light each day. Animal wellbeing and weight were monitored daily. Wild-type animals were randomly assigned to the treatment or control group. Naproxen (10 mg/kg) (Bayer, Leverkusen, Germany) or placebo (water) was orally administered from day 0 on a daily basis by oral gavage. MTX (1 mg/kg) (Emthexate; Pharmachemie BV, Haarlem, The Netherlands) or placebo (0.9% NaCl) was administered by intraperitoneal (IP) injection three times a week from day 0 onwards. After 10 weeks mice were euthanized by O₂/CO₂ asphyxiation. During further processing, the specimens were coded and the researchers thus blinded to the treatment received. One mouse in the placebo IP group died during the experiment due to complications after injection.

2.3 | (Immuno)histochemistry

Whole knee joints were fixed in phosphate-buffered formalin for 7 days, decalcified using 0.5M ethylenediaminetetraacetic acid (VWR Prolabo, Amsterdam, The Netherlands), pH 7.8, for 21 days and embedded in paraffin. Coronal issue sections of 5 μM were prepared. Before histochemistry, tissue sections on slides were deparaffinized and rehydrated using standard protocols. Sections for growth plate and cell surface measurements were stained with hematoxylin (Dako, Troy, MI). Proteoglycans were stained with Safranin-O (0.05%) (Sigma-Aldrich) and counterstained with Fast Green (0.1%) (Sigma-Aldrich). Stained sections were dehydrated and mounted in Histomount (Thermo Shandon; Thermo Fisher Scientific, Waltham, MA).

For immunohistochemical analysis of Col2a1 and Col10a1 in growth plate sections, rehydrated sections were treated with 0.4% hyaluronidase (Sigma-Aldrich). Endogenous peroxidase activity was inactivated and samples were blocked with 10% normal goat serum.

Mouse monoclonal anti-Col2a1 (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) and mouse negative control IgG1 (negative control; Dako) were used. Rabbit polyclonal anti-Col10a1 (Quartett Immunodiagnostika, Berlin, Germany) and negative control nonimmune normal rabbit serum were used. Bound antibodies were visualized with horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse secondary antibodies (EnVision+ System-HRP labeled Polymer; Dako). For detection, 3,3'-diaminobenzidine chromogen substrate (Dako) was used. Stained sections were mounted in Histomount.

2.4 | Length measurements

After dissection of the hind limb, the length of femur and tibia was measured using a digital micrometer. Hematoxylin-stained sections from similar middle regions of the growth plate of each mouse were analyzed using a Zeiss AxioScope A.1 (with AxioVision 4.8 software). The anatomical middle of the growth plates of femur and tibia was used as an internal reference point. From each growth plate, three consecutive sections were measured at the middle, a quarter, and half the distance from the anatomical reference point for the determination of the thickness of the total growth plate, the resting, proliferative and hypertrophic zones. These measurements were performed in triplicate in a randomized and blinded manner by an independent observer.

2.5 | Micro-computed tomography

After dissection of the hind leg, the growth plate region of the tibia of each sample was scanned in a micro-computed tomography (micro-CT)

(μ CT 80; Scanco Medical AG, Switzerland) at a resolution of 10 μ m (voltage 55 kVp, intensity 145 μ A, integration time 200 ms) in the air in a closed holder. Micro-CT image processing included Gauss filtering ($\sigma = 0.4$, support = 1 voxels) and segmentation of the bone phase using a global threshold of 210 per mile of the maximum gray value, corresponding to 453 mgHA/ccm. Contours were drawn manually to identify a volume of interest (VOI) of subchondral bone just distal of the growth plate with a thickness of approximately 200 microns. From the segmented images, the following morphology indices were determined: bone density, bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp).¹⁴

2.6 | Alkaline phosphatase activity and hydroxyproline assay

Enzymatic activity of alkaline phosphatase (ALP) and hydroxyproline levels were determined using a colorimetric assay as described

before.¹⁴ ALP activity was determined by measuring ALP-dependent enzymatic conversion of *p*-nitrophenyl phosphate to nitrophenyl phosphate. Hydroxyproline levels are determined by the reaction of oxidized hydroxyproline with 4-(dimethylamino)benzaldehyde. The DNA concentration in samples used for ALP and hydroxyproline assay was determined using SYBR Green I Nucleic Acid stain (Invitrogen).

2.7 | Statistics

Statistical significance ($P < .05$) was determined by the Student *t* test for in vitro experiment shown in Figure 1 and Mann-Whitney test for the in vivo experiment using the Graphpad PRISM 5.0 (La Jolla, CA). To test for normal distribution of the input data, D'Agostino-Pearson omnibus normality tests were performed. All quantitative data sets presented here passed the normality tests. Lines in graphs represent mean \pm SEM.

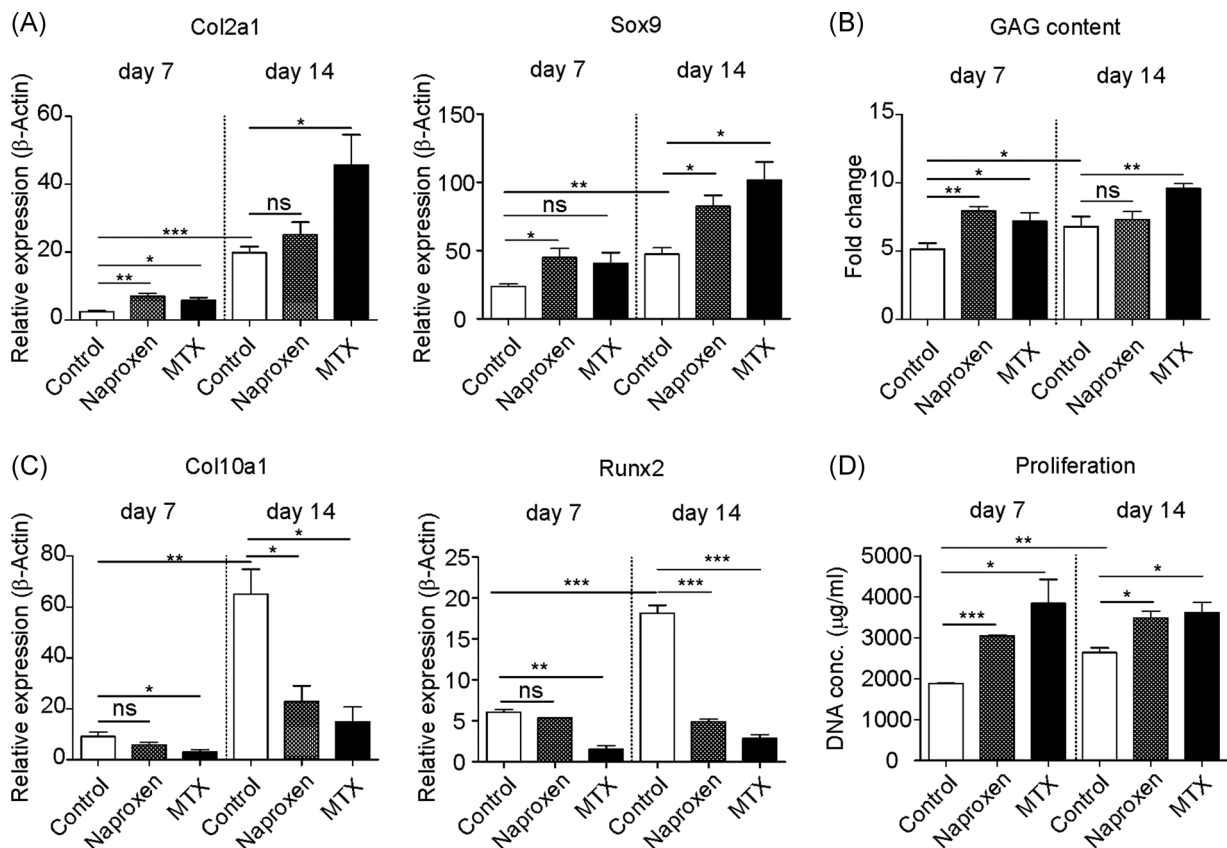


FIGURE 1 Influence of naproxen and MTX on chondrogenic differentiation of ATDC5 cells. ATDC5 cells were differentiated under control conditions (white bars) and with naproxen (1 mM; dotted bars) or MTX (1 μ M; black bars) for 7 and 14 days. A, Induction of chondrogenic markers Col2a1 and Sox9 mRNA expression was determined by RT-qPCR at day 7 and 14 in differentiation, relatively to day 0 conditions and normalized for β -actin mRNA expression. B, Glycosaminoglycan (GAG) content (corrected for protein content) was analyzed by DMB assay and fold change of $t = 7$ and $t = 14$ samples were calculated as compared to $t = 0$ samples. C, Induction of hypertrophic markers Col10a1 and Runx2 mRNA expression was determined by RT-qPCR at day 7 and 14 in differentiation, relatively to day 0 conditions and normalized for β -actin mRNA expression. D, DNA content was measured and $t = 7$ and $t = 14$ samples were compared to $t = 0$ samples. In graphs, error bars represent mean \pm SEM. Statistical significant differences ($P < .05$) are shown by an * $P < .05$, ** $P < .01$, *** $P < .0001$, NS. DMB, dimethyl methylene blue; mRNA, messenger RNA; MTX, methotrexate; NS, not significant; RT-qPCR, real-time quantitative polymerase chain reaction

3 | RESULTS

3.1 | Naproxen and MTX affect chondrogenic differentiation in ATDC5 cells

To determine if medications prescribed for JIA have the potency to alter growth plate development, we first tested if naproxen or MTX alter the course of the chondrogenic phase of endochondral ossification in vitro. Under control conditions, ATDC5 cells acquire a chondrogenic phenotype from day 7 in differentiation as determined by increased mRNA expression of collagen type II (Col2a1), sex-determining region Y box 9 (Sox9), and GAG content (Figure 1A,B; white bars).¹³ From day 14 in differentiation, these cells also express highly increased levels of chondrocyte hypertrophic marker collagen type X (Col10a1) and Runt-related transcription factor 2 (Runx2)¹³ (Figure 1C; white bars). The addition of naproxen (Figure 1; gray bars) resulted in significantly increased expression of Col2a1, Sox9, and GAG content at day 7 in differentiation. No significant alterations on Col2a1 expression and GAG content were observed at day 14 in differentiation in the presence of naproxen when compared to control conditions (Figure 1A,B). Sox9 mRNA expression was significantly increased in the naproxen-treated condition at day 14 in differentiation. At day 7 in differentiation, no significant difference was observed in the induction of hypertrophic differentiation markers Col10a1 and Runx2 after naproxen treatment. Conversely, at day 14 in differentiation naproxen treatment did result in significantly decreased induction of Col10a1 and Runx2 (Figure 1C).

Treatment of the differentiating ATDC5 cells with MTX (Figure 1A,B; black bars) resulted in increased Col2a1 (significant) and Sox9 (not significant at day 7) mRNA expression on day 7 and 14 in differentiation. Similar results were found for GAG content (Figure 1A,B). Expression of hypertrophic markers Col10a1 and Runx2 were inhibited by MTX at both days 7 and 14 (Figure 1C). Exposure of differentiating ATDC5 cells to naproxen or MTX resulted in significantly increased proliferation at day 7 and 14 (Figure 1D).

Overall, expression of chondrogenic markers (Col2a1, Sox9, and GAG content) was increased following naproxen or MTX exposure of differentiating ATDC5 cells, while hypertrophic differentiation (Col10a1 and Runx2) was attenuated as a consequence of naproxen or MTX exposure.

3.2 | Tibia and femur bone length are decreased in mice treated with naproxen or MTX

Considering the profound consequences for ATDC5 chondrogenic differentiation after naproxen or MTX exposure, we subsequently evaluated whether these compounds also alter in vivo growth plate development in mice with consequences for skeletal development. Naproxen was used at 10 mg/kg on a daily basis (which is comparable to the human dosage for JIA).¹⁵ MTX was used at 1 mg/kg/three times per week, which is at the upper limit of the human dosage used for JIA,^{16,17} but within the range of effective arthritis treatment within various mouse models.¹⁸⁻²¹ Prolonged treatment of 5-week-old skeletally immature mice with naproxen or MTX for 10 weeks did not result in significant body weight changes as compared to their control placebo-treated mice (Figure 2A). To determine if these pharmacological compounds affect longitudinal growth of the long bones, bone lengths of the tibia and femur were measured. Figure 2B,C shows that both naproxen and MTX treatment resulted in the significantly reduced bone length of the tibia and femur.

3.3 | Naproxen and MTX treatment induces abnormal growth plate development

To determine whether the impaired bone length following naproxen or MTX treatment could be attributed to disturbed growth plate development, key growth plate characteristics were evaluated. Treatment with naproxen did not result in a significant difference in the total

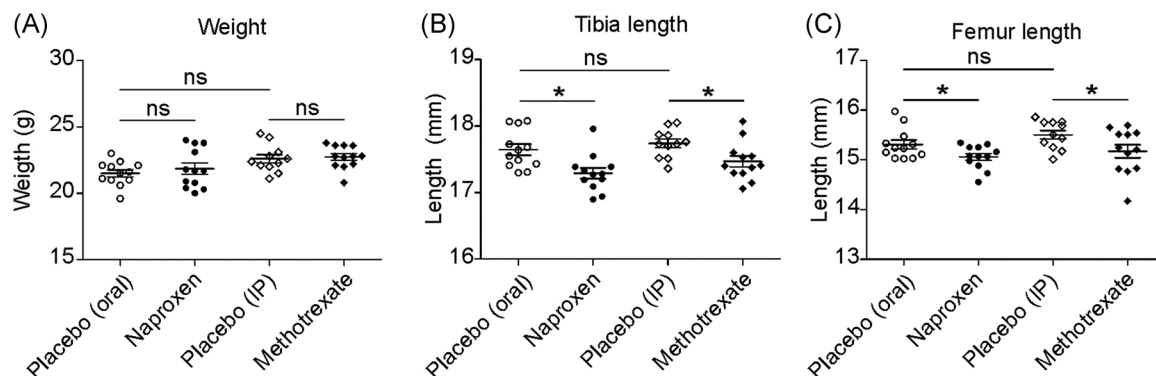


FIGURE 2 Weight and length measurements in control, naproxen, and MTX-treated mice. Five weeks old C57BL/6 mice were orally treated with either naproxen (10 mg/kg/d; n = 12) or placebo (n = 12) or received IP injections with MTX (1 mg/kg; three times per week; n = 12) or placebo (n = 11) for 10 weeks. At 15 weeks of age weight (A) and length of the tibia (B) and femur (C) were analyzed. Each dot represents the determined value for each of the individual mice and lines in graphs indicate mean \pm SEM. Statistical significant differences ($P < .05$) are shown by an *NS. IP, intraperitoneal; MTX, methotrexate; NS, not significant

thickness of the growth plate (Figure 3A), nor the resting zone thickness (Figure 3B). Interestingly, the proliferative zone of the growth plate of naproxen-administered mice was significantly thicker than in the placebo-administered mice (Figure 3C), whereas the hypertrophic zone was significantly thinner (Figure 3D) following naproxen treatment. In accordance with these results, Safranin-O and Col2a1 staining revealed macroscopically discernible higher proteoglycan and Col2a1 positivity in the growth plates of naproxen-treated mice compared to placebo-treated mice (Figure 3E,F), whereas staining intensity of hypertrophic extracellular matrix (ECM) marker Col10a1 was diminished (Figure 3G).

Treatment with MTX also resulted in abnormal growth plate development; the total thickness of the growth plate was significantly reduced as compared to the placebo (IP) mice (Figure 3A). A more detailed analysis of the different growth plate zones revealed no significant differences in the thickness of the resting zone (Figure 3B). A significantly increased thickness of the proliferative zone (Figure 3C) and a significantly thinner hypertrophic zone (Figure 3D) were found in the MTX-treated mice when compared to control placebo (IP) mice. Safranin-O and Col2a1 staining showed greater intensity in the growth plates of MTX-treated mice (Figure 3E,F), whereas Col10a1 staining intensity was slightly decreased in the MTX-treated mice (Figure 3G).

In summary, naproxen and MTX treatment affected growth plate development by altering the development of growth plate zones and ECM composition.

3.4 | Altered bone characteristics in naproxen- and MTX-treated mice

To determine if the observed aberrant development of the growth plate due to naproxen and MTX treatment resulted in a bone phenotype beyond the growth plate (compartment), bone micro-CT characteristics directly beneath the proximal tibial growth plate were determined as a measure for bone (micro) structure as a direct result of bone remodeling during endochondral ossification. Micro-CT analysis of this VOI showed a significantly lower bone volume density as a result of naproxen treatment (Figure 4A). No significant alterations in bone mineral density were observed in naproxen-treated mice as compared to placebo (oral) mice (Figure 4B). Naproxen treatment resulted in a decreased Tb.N (Figure 4C) but increased Tb.Th (Figure 4D) and increased Tb.Sp (Figure 4E). In mice treated with MTX, no significant differences were observed for bone volume density (Figure 4A) and Tb.Th (Figure 4D). Bone mineral density

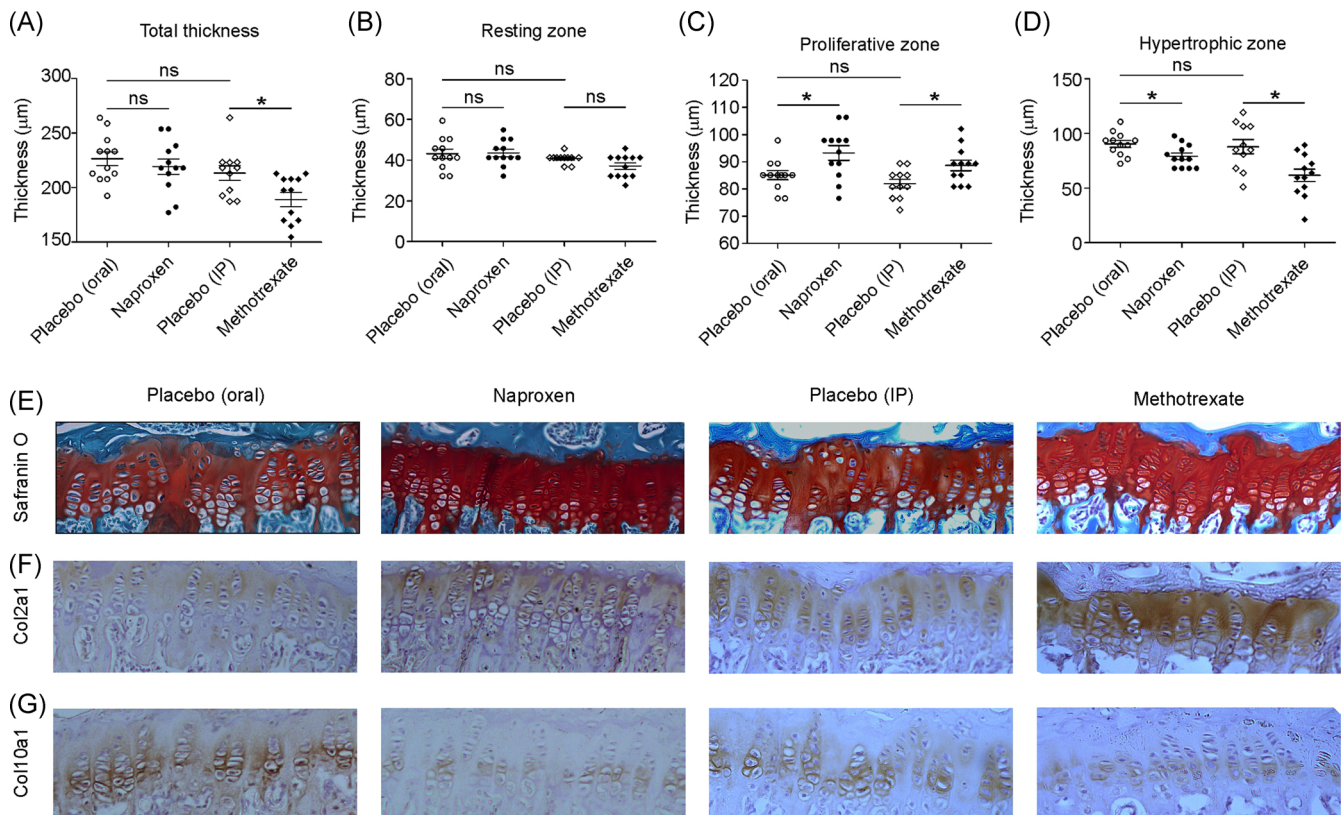


FIGURE 3 Growth plate stainings and measurements in control, naproxen- and MTX-treated mice. The thickness of the tibia growth plate and individual zones was measured on hematoxylin-stained sections. A, Thickness of the total growth plate. B, The thickness of the resting zone. C, The thickness of the proliferative zone. D, The thickness of the hypertrophic zone. In adjacent growth plate sections (immuno)histochemical stainings were performed and analyzed. E, Safranin-O/Fast green staining. F, Collagen type II immunohistochemical staining. G, Collagen type X immunohistochemical staining. Each dot represents the determined value for each of the individual mice and lines in graphs indicate mean \pm SEM. Statistical significant differences ($P < .05$) are shown by an *. NS, IP, intraperitoneal; MTX, methotrexate; NS, not significant [Color figure can be viewed at wileyonlinelibrary.com]

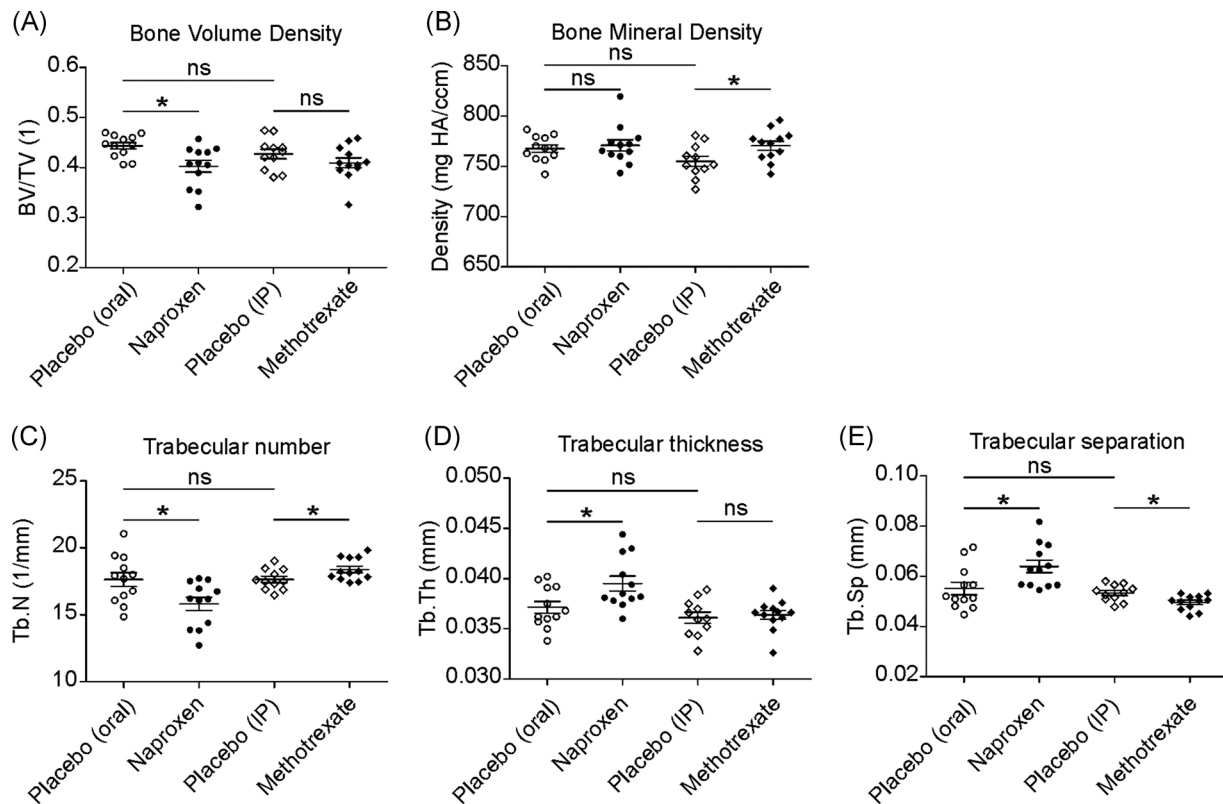


FIGURE 4 Bone microstructure analysis in control, naproxen- and MTX-treated mice. Bone microstructure in the first calcified zone in tibia was determined by μ CT. A, Quantification of bone volume density. B, Quantification of bone density. C, Quantification of trabecular thickness, number (D), and separation (E). Each dot represents the determined value for each of the individual mice and lines in graphs indicate mean \pm SEM. Statistical significant differences ($P < .05$) are shown by an *. μ CT, micro-computed tomography; IP, intraperitoneal; MTX, methotrexate; NS, not significant; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness

was significantly increased in the MTX-treated mice (Figure 4B) and this was accompanied by increased Tb.N (Figure 4C) and decreased Tb.Sp (Figure 4E). To acquire an indication of the biochemical bone quality, ALP activity and hydroxyproline content in cortical bone was measured. Treatment with naproxen resulted in increased ALP activity (Figure 5A), but a decreased hydroxyproline content in cortical bone (Figure 5B). This might indicate that the cortical bone of these mice is more brittle; as there is an increase in mineralization capacity but this is not matched with increased collagen stability. Mice treated with MTX showed significantly increased ALP activity (Figure 5A) and hydroxyproline content in the cortical bone (Figure 5B), indicative of increased bone strength. In summary, notable microstructural features were detected in the subchondral bone directly beneath the growth plate and changes in the cortical bone quality as a result of the treatment with naproxen or MTX.

3.5 | Involvement of PTHrP-Ihh loop in naproxen- and MTX-induced alterations in endochondral ossification in vitro

Multiple steps during the chondrogenic differentiation process are tightly controlled by Parathyroid hormone-related peptide (PTHrP)

and Indian Hedgehog (Ihh) through the mutual regulation of their activities.²² Ihh acts on PTHrP in a negative feedback loop to regulate early chondrocyte differentiation and the entry to hypertrophic differentiation. To further elucidate the potential biomolecular mechanism explaining the observed alterations in growth plate development as a result of naproxen or MTX treatment, gene expression in the parathyroid hormone-related peptide/Indian hedgehog (PTHrP/Ihh) pathway^{1,23} was measured in samples from the ATDC5 chondrogenic differentiation experiment shown in Figure 1. During ATDC5 chondrogenic differentiation naproxen or MTX exposure led to significant deregulation of the PTHrP/Ihh pathway as illustrated by increased PTHrP mRNA expression at day 7 and 14 in ATDC5 differentiation (Figure 6A). Conversely, expression of Ihh was decreased in naproxen and MTX conditions at days 7 and 14 in ATDC5 differentiation. In accordance with this result, the Ihh receptors patched (PTC) 1 and 2 (Figure 6B) were similarly decreased in expression by naproxen and MTX treatment at day 7 and 14 in differentiation. As a measure for functional deregulation of Ihh signaling, the expression of downstream transcription factors Gli1 and Gli3 was determined in these samples. The mRNA expression of both Gli1 and Gli3 was significantly inhibited in ATDC5 differentiation at day 7 and 14 in the naproxen- and MTX-treated cells (Figure 6C).

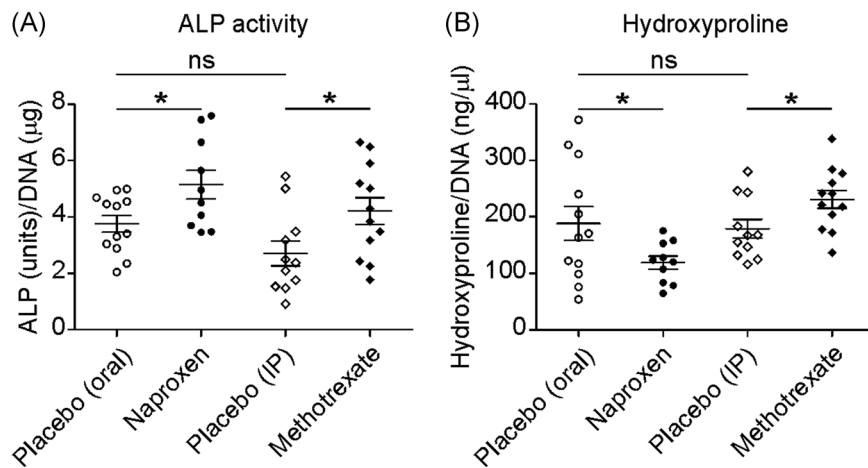


FIGURE 5 ALP activity and hydroxyproline in ulna of control, naproxen- and MTX-treated mice. Skeletal consequences of treatment with naproxen or MTX was determined. A, ALP enzymatic activity of cortical bone samples was determined and normalized to total DNA content. B, Hydroxyproline levels corrected for DNA content were determined in cortical bone samples. Each dot represents the determined value for each of the individual mice and lines in graphs indicate mean \pm SEM. Statistical significant differences ($P < .05$) are shown by an *NS. ALP, alkaline phosphatase; IP, intraperitoneal; MTX, methotrexate; NS, not significant

4 | DISCUSSION

Our previous studies^{11,14,24} determined that COX enzyme activity is involved in regulating the chondrogenic phase of endochondral ossification. Clinically, this is relevant as inhibition of COX enzymes by NSAIDs can result in hampered endochondral ossification. Following the notion that growth plate development, and thus skeletal growth, can be affected by such commonly used medications, we asked the question if growth reductions observed in JIA⁴⁻⁶ can also be attributed, at least in part, by the use of commonly used anti-inflammatory medications used in the disease management of JIA. In the present study, we used naproxen and MTX as they are frequently used medications in JIA treatment and established that they can interfere with chondrogenic differentiation both in vitro as in vivo.

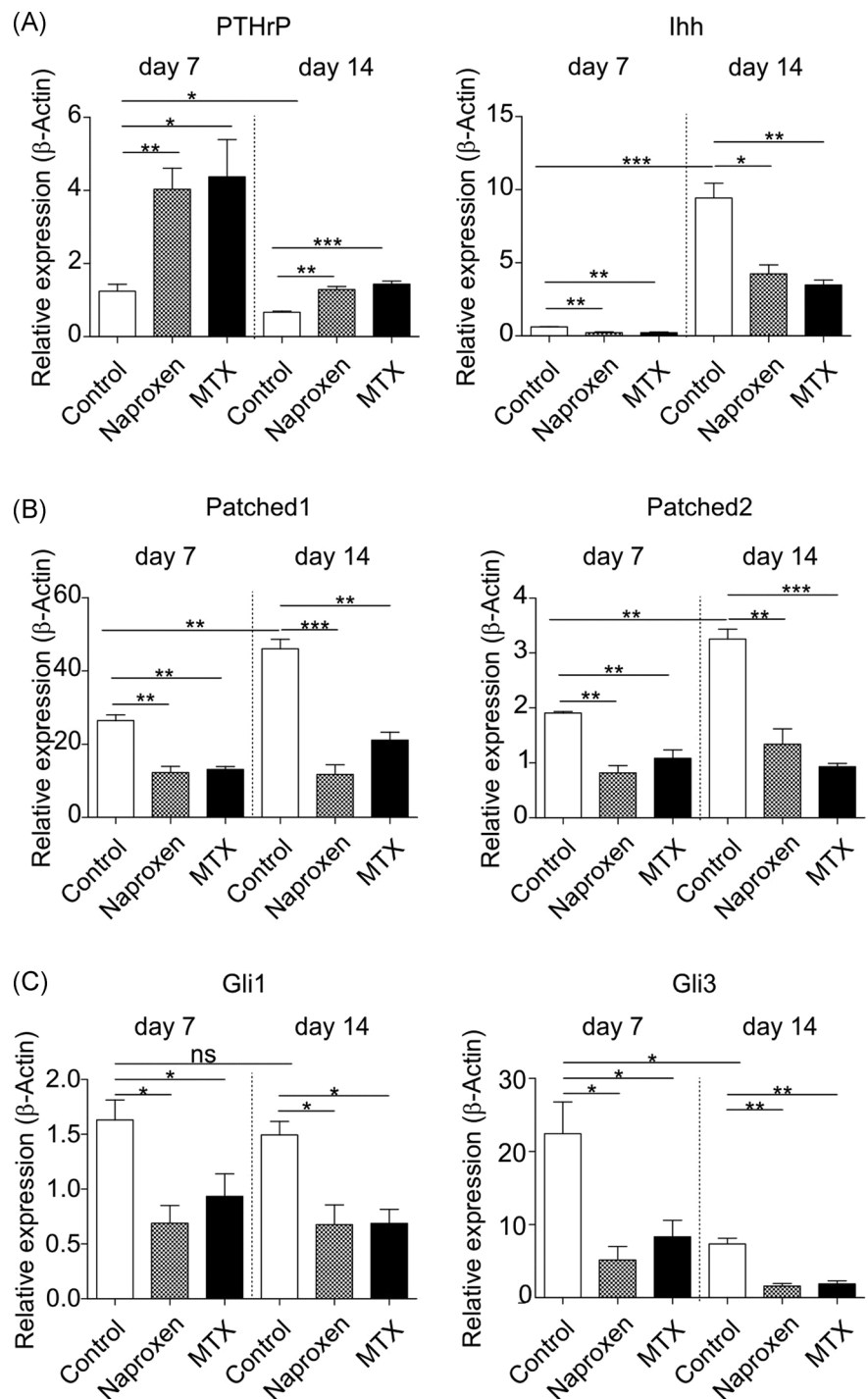
Previous studies have shown a potential for naproxen to interfere with osteogenic as well as chondrogenic mesenchymal stem cell (MSC) differentiation.^{25,26} It appears that the effect of naproxen, especially on hypertrophic gene expression in chondrogenically differentiating MSCs, is timing-dependent.^{25,26} As demonstrated by a study from Antoniou et al,²⁵ after 3 days of differentiation in the presence of naproxen the mRNA expression of hypertrophic markers such as COL10A1 and ALPL was significantly increased, whereas expression of these genes was decreased at 6 and 12 days in differentiation with naproxen exposure.²⁵ The results are in line with our findings as presented in Figure 1. Overall, mixed results are found in the literature on the effect of naproxen on chondrogenic marker expression (COL2A1, SOX9, ACAN, and GAG content) and this might be influenced by the cell model, chondrogenic differentiation induction, time of exposure to naproxen and concentration used in these studies.^{25,26} In this study, ATDC5 cells were used, which is a (murine) cell model described to resemble the chondrogenic phase of endochondral ossification.¹³ Indeed, our findings regarding the response of ATDC5 cells to naproxen, as presented in Figure 1, correspond with results from the in vivo growth plate data presented in Figure 3.

ATDC5 cells showed increased chondrogenic differentiation and proliferation combined with decreased hypertrophic differentiation when exposed to naproxen, which is validated in the mouse study; where we found increased Safranin-O and Col2a1 staining, increased proliferative zone thickness and decreased Col10a1 staining and thickness of the hypertrophic zone. Little is known about the effect of naproxen on growth plate development in vivo.²⁷ However, other studies have shown deregulation of growth plate development by other NSAIDs.^{11,24,28-30} This study is the first to show that naproxen (in an equivalent human dosage) influences murine growth plate development, the bone microstructure, cortical bone quality, and tibial and femoral bone length.

Next to naproxen, MTX, a potent inhibitor of dihydrofolate reductase and thus thymidylate and purine synthesis is frequently described to patients with JIA. MTX interferes with the synthesis and repair of DNA during cell cycle replication and has anti-inflammatory properties.³¹ The effect of MTX on chondrocytes or MSC differentiating into the chondrogenic lineage is not well studied, but it is reported that MTX does not affect the survival, proliferation, and functional characteristics of cultured MSCs.³² In addition, Kastrinaki et al³³ showed that bone marrow-derived MSCs from adult patients with rheumatoid arthritis have impaired proliferative and clonogenic capacity when compared to healthy bone marrow-derived MSCs, but this was not correlated to previous MTX treatment of these patients. Our results demonstrate an increased chondrogenic potential and decreased hypertrophic phenotype in differentiating ATDC5 cells when exposed to MTX, accompanied by an increase in proliferation during differentiation (Figure 1), showing that MTX does interfere with the endochondral ossification pathway.

In vivo, we observed a decreased tibia and femur length after 10 weeks of treatment with MTX (1 mg/kg/three times a week), and this was accompanied by significant changes in bone microarchitecture, cortical bone quality and growth plate thickness and composition (Figures 2-4). These results are mostly in line with Iqbal et al,³⁴ who reported reduced length of the long bones which was accompanied

FIGURE 6 Involvement of Ihh-PTHrP loop in effects of naproxen and MTX on endochondral ossification in vitro and in vivo. ATDC5 cells were differentiated under control conditions (white bars) and with naproxen (1 mM; dotted bars) or MTX (1 μ M; black bars) for 7 and 14 days. A, PTHrP and Ihh mRNA expression were determined by RT-qPCR at day 7 and 14 in differentiation, relatively to day 0 conditions and normalized for β -actin mRNA expression. B, mRNA expression of Ihh receptor patched 1 and 2 was determined by RT-qPCR at day 7 and 14 in differentiation, relatively to day 0 conditions and normalized for β -actin mRNA expression. In graphs, error bars represent mean \pm SEM. Statistical significant differences ($p < .05$) are shown by an *** $p < .01$, ** $p < .0001$, NS, mRNA, messenger RNA; MTX, methotrexate; NS, not significant; RT-qPCR, real-time quantitative polymerase chain reaction



by the reduced height of the growth plate in mice treated with MTX (3.5 mg/kg/every second day for 3 weeks). Other studies in rats and rabbits have also reported suppression in growth plate development and skeletal growth after treatment with various doses and treatment regimens of MTX.^{35,36} There are however differences between these studies, including our own, regarding the specific effect of MTX on growth plate composition, specifically concerning proliferation/proliferative zone, and trabecular microstructures and bone mineral density.^{35,36} It is likely that these differences can be attributed to the

animal model, skeletal age when treatment is started, treatment duration, and concentration of MTX, indicating that more research is needed to fully investigate the effect of MTX on skeletal development and the influence of above-mentioned parameters. However, this is the only study that studies the influence of the medications on endochondral ossification in both in vitro and in vivo. The effects of naproxen and MTX on growth plate morphology are very similar, but those on the character of bone architecture beneath the growth plate (Figure 4) and cortical bone (Figure 5) are different.

As metaphyseal bone structure formation not only relies on endochondral ossification but also on bone remodeling pathways, it is expected that these medications influence this process in a different manner. Current literature is however inconclusive about the effect of naproxen³⁷ or MTX³⁸ on bone remodeling and this is worth investigating further.

Multiple steps during the chondrogenic differentiation process are tightly controlled by PTHrP and Ihh through the mutual regulation of their activities. Ihh acts on PTHrP in a negative feedback loop to regulated early chondrocyte differentiation and the entry to hypertrophic differentiation.¹ Upon binding of Ihh on the PTC receptor, smoothed is no longer suppressed, and this results in the activation of the Gli transcription factor family. These Gli zinc-finger transcription factors are responsible for Ihh-induced lineage commitment of MSCs, induction of chondrocyte hypertrophy, and can be used as markers for the Ihh signaling activity.³⁹ PTHrP shares the same receptor as PTH and is also described to influence the chondrogenic differentiation of MSCs.⁴⁰ Interestingly, for naproxen it was already described that its effects on hypertrophic differentiation during osteogenic and chondrogenic differentiation of MSCs could be explained via deregulation of the Ihh/PTHrP signaling.^{25,26} Amano et al,⁴¹ showed that Ihh regulates expression of Col10a1 directly via binding of the Gli1/2 transcription factors on the Col10a1 promoter, but also indirectly via induction of Runx2 and subsequent transcriptional complex formation of Gli1/2, Runx2, and Smads which are able to induce Col10a1 expression. It is thus likely that the observed decreased hypertrophic differentiation (Col10a1, Runx2; Figure 1) might be due to deregulated Ihh signaling. Indeed, Figure 5 shows decreased Ihh expression, as well as decreased expression of its receptor and the Gli transcription factors. NSAIDs, such as naproxen, mediate their action via inhibition of COX enzyme activity which diminishes the biosynthesis of prostaglandins (PGs).⁴² Indeed, a connection between PG signaling and PTHrP has been proposed before during osteogenesis.⁴³ In addition, from literature and our own previous studies, it is known that the BMP/Smad1/5/8 signaling axis and subsequent induction of hypertrophic differentiation are sensitive to PGE2 regulation.^{11,44} This opens the possibility that the observed chondrogenic/hypertrophic alterations due to naproxen can be attributed to inhibition of PGs and subsequent BMP-induced Smad1/5/8 activation.

Unfortunately, not much is known on how MTX affects major signaling pathways in endochondral ossification. However, Olesen et al⁴⁵ showed a concentration-dependent decreased hedgehog signaling, as indicated by Gli1 and PTC1 gene expression, after MTX treatment of basal cell carcinoma cells compared to immortalized keratinocytes. In addition, MTX-induced bone loss in rats is mediated by attenuated Wnt/b-catenin signaling; a pathway described to interact with the Ihh signaling and likewise important in regulating hypertrophic differentiation.^{46,47} These data are in line with the findings presented in Figure 5 where MTX resulted in decreased Ihh signaling and hypertrophic differentiation. Cell culture models such as ATDC5 or chondrogenic differentiation of MSCs *in vitro* are synchronized events, where all cells simultaneously differentiate from a progenitor cell towards a chondrogenic and eventually hypertrophic state. This in

vitro simultaneous differentiation cannot fully recapitulate the particular spatiotemporal nature of the Ihh/PTHrP feedback loop as it is active *in vivo*. Decreased Ihh signaling in hypertrophic chondrocytes *in vivo* would theoretically result in decreased PTHrP expression and thus a shorter proliferative zone. This is not observed in this study. Consequently, future studies should focus on identifying the interaction between JIA medications and the Ihh/PTHrP feedback loop *in vivo*, to clarify the role of the Ihh/PTHrP feedback loop in the here observed effects on endochondral ossification.

An alternative explanation for the observed effects on hypertrophic differentiation *in vitro* and the hypertrophic zone and subchondral bone *in vivo* can be found in vascular endothelial growth factor (VEGF) signaling. VEGF expression, essential for vascularization of the hypertrophic zone and bone remodeling during endochondral ossification, is under the tight control of COX-2 and a direct target of Wnt/b-catenin signaling.^{48,49} It might, therefore, be possible that COX-2 inhibition by naproxen or Wnt/b-catenin attenuation by MTX results in abnormal VEGF expression and vascularization of the hypertrophic zone of the growth plate and subchondral bone leading to disrupted growth plate remodeling which is seen in this study. The increased chondrogenic marker expression (Col2a1 and Sox9 expression, GAGs content, Safranin-O staining) after either naproxen or MTX treatment was seen in this study might also be explained by deregulated PTHrP/Ihh signaling. In Figure 6, we observed a significant upregulation of PTHrP in the treatment conditions, which is most pronounced at day 7 in ATDC5 differentiation. In line with previous literature, increased PTHrP is shown to increase chondrogenic marker expression such as Col2a1 in MSCs which might explain our observed effects.^{40,50}

Overall, these results show that naproxen and MTX have a profound action on endochondral ossification during growth plate development, aberrant subchondral bone morphology, and reduced bone length. A better understanding of how these medications affect growth plate development will improve the insight in endochondral ossification and will reveal possibilities to improve the treatment of pediatric patients.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Substantial contributions to research design: MC, AW, and TW. Substantial contributions to the acquisition of samples: MC and TC. Substantial contributions to analysis: MC, TC, BvR, and MH.

Substantial contributions to the interpretation of data: MC, TC, AW, TW, BvR, and MH. Drafting of the paper: MC and AW. Revising paper critically: MC, TC, BvR, MH, LWR, AW, and TW. Approval of the submitted and final versions: MC, TC, BvR, MH, LWR, AW, and TW. All authors have read and approved the final submitted manuscript.

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