Interferon Gamma, but not Calcitriol Improves the Osteopetrotic Phenotypes in ADO2 Mice

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Running Title: Interferon gamma improves the osteopetrotic phenotypes in ADO2 mice

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Abstract

ADO2 is a heritable osteosclerotic disorder that usually results from heterozygous missense dominant negative mutations in the chloride channel 7 gene (*CLCN7*). ADO2 is characterized by a wide range of features and severity, including multiple fractures, impaired vision due to secondary bony overgrowth and/or the lack of the optical canal enlargement with growth, and osteonecrosis/osteomyelitis. The disease is presently incurable, although anecdotal evidence suggests that calcitriol and interferon gamma-1b (IFN-G) may have some beneficial effects. To identify the role of these drugs for the treatment of ADO2, we utilized a knock-in (G213R mutation in *Clcn7*) ADO2 mouse model that resembles the human disease. Six-week-old ADO2 heterozygous mice were administered vehicle (PBS) or calcitriol or IFN-G 5 times per week for 8 weeks. We determined bone phenotypes using DXA and μCT, and analyzed serum biochemistry and bone resorption markers. ADO2 mice treated with all doses of IFN-G significantly ($p<0.05$) attenuated the increase of whole body aBMD and distal femur BV/TV gain in both male and female compared to the vehicle group. In contrast, mice treated with low and medium doses of calcitriol showed a trend of higher aBMD and BV/TV whereas high dose calcitriol significantly ($p<0.05$) increased bone mass compared to the vehicle group. The calcium and phosphorus levels did not differ between vehicle and IFN-G or calcitriol treated mice; however, we detected significantly ($p<0.05$) elevated levels of CTX/TRAP5b ratio in IFN-G treated mice. Our findings indicate that while IFN-G at all doses substantially improved the osteopetrotic phenotypes in ADO2 heterozygous mice, calcitriol treatment at any dose did not improve the phenotype and at high dose further increased bone mass. Thus, use of high dose calcitriol therapy in ADO2 patients merits serious reconsideration. Importantly, our data support the prospect of a clinical trial of IFN-G in ADO2 patients.

Keywords: Bone mineral density; ADO2; Calcitriol; Interferon-gamma; Osteopetrosis

Introduction

Autosomal Dominant Osteopetrosis type II (ADO2) is an osteosclerotic bone disease that usually results from heterozygous missense dominant negative mutations in the chloride channel 7 (*CLCN7*) gene[1,2]. ADO2 is characterized by a wide range of features and severity, including extensive osteosclerosis, especially of the skull base, pelvis and vertebrae, multiple fractures, impaired vision and hearing, osteomyelitis and hematologic failure[3-5]. ADO2 patients develop impaired vision due to secondary bony growth and/or failure of the optical canal to enlarge with growth. The osteomyelitis in these patients results from poor bone resorption, less vascularization and, possibly, increased propensity to infection due to decreased superoxide production. Also, hematologic failure is due to loss of marrow cavity because excessive bone accumulation takes the place of marrow, which can lead to extramedullary hematopoiesis in severe cases of ADO2. The disease is presently incurable, although anecdotal evidence suggests that calcitriol and interferon gamma-1b (IFN-G) may have some beneficial effects.

High dose calcitriol has been tried for the first time with limited success in a single case report for the treatment of the severe malignant form of osteopetrosis in 1984[6]. This treatment is still being used for ADO2 patients based on this early evidence (OMIM #166600) as well as the possibility that calcitriol might potentially stimulate osteoclastic bone resorption[26,27] (Econs, unpublished observations). In addition, the FDA approved drug, recombinant human interferon gamma-1b (Actimmune®) has been used in osteopetrotic patients with the recessive form of the disease[7,8]. However, currently no evidence exists indicating that either calcitriol or IFN-G has any beneficial effect in the less severe dominant form of osteopetrosis, ADO2.

We developed two knock-in (p.G213R) mouse models of ADO2 that has a missense mutation in the *Clcn7* gene[9], which is analogous to one of the common mutations found in humans[5]. The

mutation led to severe osteopetrosis and lethality in homozygous mice but produced substantial phenotypic variability in heterozygous mice on different genetic backgrounds that resemble the human disease of ADO2[9]. We also found that mice with the heterozygous mutation on 129sv background exhibited moderately severe bone phenotypes with increased whole body aBMD and BMC as well as an almost two-fold increase of trabecular bone volume at the distal femur in both male and female[9]. Also, these mutant mice had high number of poorly resorbing osteoclasts.

In this study, we exploited the unique animal model of ADO2 on the 129 background that was developed in our laboratory to identify the effect of both calcitriol and IFN-G drugs for the treatment of ADO2. Our findings indicate that while IFN-G at different doses substantially improved the osteopetrotic phenotypes in both male and female ADO2 heterozygous mice, calcitriol treatment at any dose did not improve the bone phenotypes and at high dose even further exacerbated the osteosclerotic condition with increased bone mass.

Materials and Methods

Reagents

We purchased calcitriol (1α,25-Dihydroxyvitamin D3) from Sigma (Cat # D1530) as a powder form, which was dissolved in a mixture of 100% ethanol and sterile PBS at different concentrations in small aliquots to avoid multiple freezing and thawing, and stored at -20°C until needed for use. As calcitriol is light sensitive, we stored it in the dark. We obtained mouse IFN-gamma from R&D Systems (Cat # 485-MI) as a lyophilized form which was reconstituted in sterile PBS containing 0.1% bovine serum albumin at different concentrations in small aliquots, and stored at -20°C until used.

Experimental Animals

We utilized 80 ADO2 heterozygous mice (10 mice per sex and per treatment group) on 129 background[9] in the calcitriol study. Mice were randomly assigned to vehicle, low (0.1 μg/kg), medium (0.5 μg/kg) and high (1μg/kg) dose groups. At 6 weeks of age ADO2 mice received either vehicle (PBS) or different doses of calcitriol intraperitoneally (IP) 5 times per week for the duration of 8 weeks. We performed two different studies involving interferon-gamma (IFN-G): phase I and II. For each of these studies, we utilized 80 ADO2 heterozygous mice on 129 background[9] (10 mice per sex and per treatment group). For the phase I IFN-G study, mice received either vehicle (PBS), low (1.5 μg/kg), medium (7.5 μg/kg) and high (37.5 μg/kg) doses of IFN-G subcutaneously (SC) 3 times per week for 8 weeks. For phase II IFN-G study, mice were administered with either vehicle (PBS), low (22.5 μg/kg), medium (37.5 μg/kg) and high (100 μg/kg) doses of IFN-G SC 5 times per week for the duration of 8 weeks.

All mice were generated and maintained at Indiana University. Mice were housed in polycarbonate cages in a vivarium maintained on a 12-h light and 12-h dark cycle and were fed a regular diet and water *ad libitum*. The procedures performed throughout the experiment followed the guidelines of the Indiana University Animal Care and Use committee (IACUC).

Euthanasia and specimen collection

Mice were euthanized at 14 weeks of age, and lower limbs were dissected from these animals. The femora were stripped of muscle, transferred to 70% ethyl alcohol and stored at 4ºC for micro-CT analyses. We collected blood within 2 hours of last IFN-G and calcitriol injections by cardiac bleeding. We collected urine at the time of sacrifice through puncture of the urinary bladder. Blood samples were allowed to clot for about 1 hour at room temperature and serum was collected by centrifugation and stored immediately at -80°C until analysis. Urine samples were immediately preserved at -80°C until analysis.

Dual energy X-ray absorptiometry (DXA)

The whole body was scanned using DXA (PIXImus II mouse densitometer; Lunar Corp., Madison, WI, USA) with ultra-high resolution (0.18 × 0.18 mm/pixel) at 14 weeks of age. The machine was calibrated prior to each DXA scanning session using a phantom supplied by the manufacturer. The whole body scans were performed with the mice in a prone position, with each limb spread on a plastic tray. The global window was defined as the whole-body image minus the calvarium, mandible, and teeth from which whole body aBMD (g/cm^2) and BMC (g) measurements were obtained.

Micro Computer Tomography (μCT) analysis

The femurs were scanned with a high resolution μCT scanner (vivaCT 40, Scanco Medical AG, Brüttisellen, Switzerland) with an isotropic voxel size of 10.5 μm^3. Before scanning, the CT-scanner was calibrated using a phantom according to the manufacturer's recommendation. From the scout-view, the growth plate location was identified and trabecular bone measurements consisting of 200 slices (2.1 mm) were done from about 1 mm below the growth plate as described previously[9]. Finally, 3D and 2D morphometric evaluations were performed for the trabecular bone from each scan, and bone volume over tissue volume (BV/TV) and structural parameters (trabecular number; Tb.N, trabecular thickness; Tb.Th and trabecular separation; Tb.Sp) were determined. We chose to investigate long bone instead of other skeletal regions due to simplicity and reproducibility of the measurements involved. In addition, we found the skeletal phenotypes of ADO2 mice correlates well in both long bone and vertebrae[9].

Serum biochemistry and biomarkers

Serum calcium, phosphorus and alkaline phosphatase were measured using the Randox Rx kit (Daytona Analyzer, WV, USA). In addition, mouse serum levels of IFN-G (R&D systems, MN, USA), CTX and TRAcP5b isoform (Immunodiagnostics Systems, AZ, USA) were measured by Enzyme-Linked ImmunoSorbent (ELISA) kits according to the manufacturers' instructions. We used mouse IFN-gamma Quantikine ELISA kit (MIF00) for IFN-G assay, RatLaps^{TM} (CTX-1) EIA kit (AC-06F1) for CTX and MouseTRAP^{TM} (TRAcP 5b) ELISA kit (SB-TR103) for TRAcP5b assay. We used singleton for IFN-G and TRAPc5b assays, and duplicates for CTX assay. The percent of coefficient of variation (% CV) was within the range (<10%) reported for the CTX kit.

Urine analysis

Urine levels of calcium, phosphorus and creatinine were measured using the Randox Rx kit (Daytona Analyzer, WV, USA). Urine calcium level was normalized by urine creatinine level to measure calcium creatinine ratio (Ca/Cr).

Statistical analysis

Quantitative data were expressed as mean ± SEM. Statistical differences were identified by one-way analysis of variance (ANOVA) test using the statistical software package StatView (Abacus Concepts, Inc., Berkeley, CA). Fisher's protected least significant difference (PLSD) was used for all pairwise post hoc comparisons. The level of significance was set at $p \leq 0.05$.

Results

Calcitriol treatment did not improve the whole body aBMD and BMC phenotypes in ADO2 mice

Male ADO2 mice treated with low and medium doses of calcitriol for 8 weeks demonstrated no significant differences for aBMD whereas the group treated with high dose calcitriol exhibited

significantly higher aBMD (5%; p=0.02) as compared to the vehicle group (Figure 1and Supplemental Table 1). Female ADO2 mice treated with all doses of calcitriol showed similar values for whole body aBMD. In addition, both male and female mice treated with all doses of calcitriol exhibited no differences for BMC compared to the vehicle mice.

Calcitriol treatment did not attenuate the trabecular bone mass gain in ADO2 mice

Compared to the vehicle-treated males, mice treated with low and medium doses of calcitriol displayed no significant differences for any trabecular bone morphometry parameters after 8 weeks of treatment (Figure 1and Supplemental Table 1). In contrast, the high dose calcitriol significantly increased BV/TV (20%; p=0.01) and Tb.Th (9%; p=0.002) in male mice as compared to the vehicle treated group. Female mice treated with all doses of calcitriol showed similar values for BV/TV, Tb.N, Tb.Th and Tb.Sp as compared to the vehicle group.

Serum biochemistry and biomarkers were similar between vehicle and calcitriol-treated ADO2 mice

Serum levels of calcium and phosphate were similar among vehicle and multiple doses of calcitriol-treated ADO2 mice at the end of the 8-week treatment (Table 1). A trend toward lower level of CTX was observed in male mice; however, the level of TRAPc5b was similar, resulting in lower CTX/TRAPc5b ratio in male ADO2 mice (Supplemental Table 1 and supplemental figure 1). The female ADO2 mice demonstrated similar levels for all of these measurements among vehicle and calcitriol-treated groups.

Urine calcium level was higher in high dose calcitriol-treated ADO2 mice

The urinary calcium level was 3-fold higher in both male and female high dose calcitriol-treated mice as compared to the vehicle group (Supplemental figure 2). Similarly, the urine calcium

creatinine ratio (Ca/Cr) was 3-fold higher in both male and female ADO2 mice treated with high dose calcitriol as compared to the vehicle group. No differences for urinary calcium and Ca/Cr were observed between vehicle and calcitriol-treated ADO2 mice with low and medium doses in both male and female.

Phase I - IFN-G study:

High dose IFN-G significantly improved the bone phenotypes in ADO2 mice

For the phase I IFN-G study, male and female ADO2 mice received either vehicle, low (1.5 μg/kg), medium (7.5 μg/kg) or high (37.5 μg/kg) doses of IFN-G 3 times per week for 8 weeks. Serum levels of calcium and phosphate were similar among vehicle and different doses of IFN-G-treated ADO2 mice at the end of the treatment (Table 1). Vehicle treated wild-type and ADO2 mice showed very small but detectable amount of serum IFN-G (Supplemental Table 2). Low dose IFN-G increased the serum level 130 times in male and 50 times in female, whereas medium and high doses of IFN-G raised it 5x and 35x further compared to the low dose group. High dose IFN-G significantly lowered whole body DXA (aBMD; p=0.0008 and BMC; p=0.003) and distal femur μCT (BV/TV; p=0.05, and Tb.Th; p=0.003) parameters in male ADO2 mice (Supplemental Table 2). In addition, male ADO2 mice in the high dose IFN-G group exhibited significantly higher serum CTX (p=0.05), lower serum TRAPc5b (p=0.05) and higher CTX/TRAPc5b ratio (p=0.002) as compared to the vehicle mice. Female ADO2 mice treated with high dose IFN-G also showed significantly lower serum TRAPc5b (p=0.002) and higher CTX/TRAPc5b ratio (p=0.05) compared to the vehicle group (Supplemental Table 2).

Phase II - IFN-G study:

Serum IFN-G level measurement

For the phase II IFN-G study, mice were administered with either vehicle (PBS), low (22.5 μg/kg), medium (37.5 μg/kg) or high (100 μg/kg) doses of IFN-G SC 5 times per week for 8 weeks. Vehicle treated wild-type and ADO2 mice exhibited very small but detectable amounts of serum IFN-G in phase II IFN-G study (Supplemental Table 3 and supplemental figure 3). Low dose IFN-G administration in ADO2 mice raised the serum level of IFN-G almost 2000 times in male and 1300 times in female compared to the vehicle treated groups for phase II study. Also, medium and high doses of IFN-G further increased the serum levels of IFN-G by 1.4-1.7x and 4.6-7.7x, respectively, when compared to the low dose group (Supplemental figure 3).

IFN-G at all doses improved the whole body aBMD and BMC phenotypes in ADO2 mice

Male mice treated with all doses of IFN-G showed significantly lower aBMD as compared to the vehicle treated mice (Figure 2 and Supplemental Table 3). At the end of 8 weeks of treatment, both the low (22.5 μg/g) and medium (37.5 μg/g) doses of IFN-G reduced aBMD by 7% ($p=0.0001$ and $p=0.001$, respectively) whereas high dose of IFN-G (100 μg/g) decreased aBMD by 6% ($p=0.004$) in male ADO2 mice. Female mice treated with low dose IFN-G also significantly lowered aBMD by 7% ($p=0.004$) whereas medium and high doses of IFN-G treated female mice demonstrated a trend of decreased aBMD by 3-4% as compared to the vehicle treated group. Both low and medium doses of IFN-G reduced the whole body BMC in male ADO2 mice (14%; $p=0.003$ and 13%; $p=0.006$, respectively) and there was a trend of lower (8%) BMC in the high dose IFN-G treated group. Also, all doses of IFN-G treated female mice showed a trend of lower BMC (5-7%) relative to the vehicle mice (Figure 2 and Supplemental Table 3).

IFN-G at all doses attenuated the trabecular bone mass gain in ADO2 mice

Compared to the vehicle treated mice, male ADO2 mice treated with low and medium doses of IFN-G displayed significantly lower BV/TV (23%; p=0.05 and 24%; p=0.04, respectively) (Figure 3B and Supplemental Table 3). High dose IFN-G produced a trend of lower BV/TV (17%) in male mice. Female ADO2 mice treated with all doses of IFN-G showed significantly lower BV/TV (low dose, 34%, p=0.001; medium dose, 24%, p=0.02; high dose, 32%, p=0.002, respectively) relative to the vehicle treated mice. While male ADO2 mice treated with all doses of IFN-G showed a trend of lower Tb.N (9-14%) compared to the vehicle group, female mice treated with low dose IFN-G exhibited significantly lower Tb.N (20%, p=0.03) whereas medium and high doses of IFN-G mice showed a trend of lower Tb.N (8-16%) relative to the vehicle group. In addition, Tb.Th was 8-13% lower in male mice treated with all doses of IFN-G, whereas this value was significantly lower in female ADO2 mice at all doses (low dose, 19%, p=0.0001; medium dose, 16%, p=0.0001; high dose, 18%, p=0.0001, respectively) relative to the vehicle mice (Figure 3B and Supplemental Table 3).

IFN-G at all doses improved the serum biomarker phenotypes in ADO2 mice

Serum levels of calcium and phosphate were similar among vehicle and different doses of IFN-G-treated ADO2 mice at the end of the treatment (Table 1). IFN-G treatment at all doses significantly increased CTX levels (low dose, 72%, p=0.0001; medium dose, 53%, p=0.0001; high dose, 50%, p=0.001, respectively) in male ADO2 mice (Figure 4 and Supplemental Table 3). Male mice treated with low dose IFN-G exhibited a trend of lower (16%) TRAPc5b and medium dose IFN-G treated male mice showed significantly lower (33%; p=0.05) TRAPc5b as compared to the vehicle group. In female, low dose IFN-G treatment significantly lowered the TRAPc5b (34%; p=0.01) level while both medium and high dose groups demonstrated a trend of lower levels of TRAPc5b (25-26%) relative to the vehicle group. The CTX/TRAPc5b ratio was significantly higher in male mice treated with all doses of IFN-G (low dose, 109%, p=0.01; medium dose, 133%, p=0.001; high dose, 81%, p=0.05,

respectively). Female mice treated with all doses of IFN-G also exhibited a trend of a higher CTX/TRAPc5b ratio (50-75%) when compared to the vehicle mice (Figure 4 and Supplemental Table 3).

Discussion

In this study, we demonstrated that IFN-G at all doses substantially improved the osteopetrotic phenotypes by significantly attenuating the increase of whole body aBMD and distal femur BV/TV gain in both male and female ADO2 heterozygous mice. In addition, serum levels of CTX/TRAPc5b ratio were significantly higher in IFN-G treated mice as compared to the vehicle group. In contrast, calcitriol treatment at any dose did not improve the phenotype and further increased bone mass at the highest dose when compared to the control group.

Calcitriol, particularly at a high dose, has been used for the treatment of the severe form of osteopetrosis with inconsistent results. While Key L. et al.[6] found that three months of high-dose calcitriol therapy increased bone turnover and improved disease severity in an infant with malignant recessive osteopetrosis, in another study[10] treatment of malignant infantile osteopetrosis with this regimen failed to show any clinical, radiological or histological improvement. In contrast to recessive osteopetrosis, there is no published evidence for the beneficial effect of calcitriol in the patients with the dominant form of the disease. However, this therapy is still being used in ADO2 patients based on the anecdotal evidence as well as cell culture studies[26,27] indicating that calcitriol has the potential to stimulate bone resorption involving osteoclasts. We treated our ADO2 heterozygous mice with low, medium and high doses of calcitriol 5 times per week for the duration of 8 weeks. The high dose (1 μg/kg) used in this study is 5-10 times higher than the clinical dose of calcitriol routinely used in patients. However, calcitriol with all three doses failed to show any improvement of the osteopetrotic

phenotype in either male or female ADO2 mice (Figure 1and Supplemental Table 1). Moreover, male mice treated with high dose calcitriol exhibited significantly higher aBMD, BV/TV and Tb.Th as compared to the vehicle group, strongly indicating that calcitriol has no beneficial effect in the treatment of ADO2 (Figure 1and Supplemental Table 1).

Several studies have demonstrated that IFN-G stimulated osteoclast formation and superoxide production in osteoclasts[7,8,11,12]. A six-month therapy with IFN-G in severe recessive osteopetrosis patients decreased trabecular bone, which was maintained for 18 months[8]. In addition, IFN-G treatment of osteoclasts derived from the malignant osteopetrosis patients resulted in formation of larger osteoclasts with increased production of superoxide compared to the normal control osteoclasts[11]. Although, these studies were performed in osteopetrotic patients with the recessive form of the disease, these data also suggest that IFN-G therapy might provide beneficial effect in the dominant form of osteopetrosis.

Previous studies demonstrated conflicting evidence whether IFN-G stimulates or inhibits osteoclasts and thereby influences bone resorption. Duque G. et al.[13] demonstrated that IFN-G knockout mice had an osteoporotic phenotype accompanied with decreased bone formation and resorption markers. In addition, the same authors' found that administration of IFN-G in ovariectomized mice significantly improved bone mass and architecture[13]. They either administered 2000 or 10,000 IU (equivalent to approximately 2 or 10 μg) of IFN-G per day IP three times per week in 8-week-old female B6 mice for 6 weeks. Along this line, several cell culture studies demonstrated that IFN-G inhibited osteoclastogenesis through multiple mechanisms such as inhibition of osteoclast proliferation[14], reduction of Ctsk mRNA and protein expression[15,16], inhibition of RANKL-RANK pathway[17,18], and suppression of Rankl-induced expression of Nfatc1, NF-kB and JNK pathways in osteoclasts[17-20]. In contrast, the pro-osteoclastogenesis effect due to IFN-G treatment was

demonstrated by multiple studies mediating mainly through the stimulation of superoxide production[7,12,21-23]. IFN-G had also been shown to stimulate osteoclast formation and bone loss via antigen-derived T cell activation[24] and by induction of pro-osteoclast fusion[25]. Therefore, a clear need for more data in an appropriate animal model was necessary before this drug could be considered for a clinical trial in ADO2 patients. Our ADO2 mice on 129 background that resemble the human disease of ADO2 [9] provided us the opportunity to test this drug in vivo.

In the phase I IFN-G study, we treated male and female ADO2 mice with low (1.5 μg/g), medium (7.5 μg/g) and high (37.5 μg/g) doses of IFN-G three times per week for 8 weeks. The low dose is similar to the dose for patients with chronic granulomatous disease or severe osteopetrosis. The medium and high doses are 5- and 25-fold higher than that used in humans. We identified that the high dose IFN-G significantly lowered aBMD, BMC, BV/TV, Tb.N and Tb.Th in male ADO2 mice (Supplemental Table 2). In addition, male ADO2 mice in the high IFN-G dose group exhibited significantly higher serum CTX (a measure of osteoclast activity), lower serum TRAPc5b (an indication of osteoclast number) and higher CTX/TRAPc5b ratio (represents osteoclast activity or bone resorption normalized by osteoclast number) as compared to the vehicle treated mice. Female ADO2 mice treated with high dose IFN-G also displayed significantly lower serum TRAPc5b and higher CTX/TRAPc5b ratio compared to the vehicle group (Supplemental Table 2). Subsequently, considering the higher rate of mouse metabolism when compared with humans which could shorten the half-life of the IFN-gamma, we modified the doses and frequency of IFN-G administration in our phase II IFN-G study as follows: low (22.5 μg/g), middle (37.5 μg/g) and high (100 μg/g) doses five times per week for the duration of 8 weeks. The low dose (22.5 μg/kg; 5 times/week) for phase II study is equivalent to the high dose (37.5 μg/g; 3 times/week) of phase I study, which is 25-fold higher

than that used in humans. The medium and high doses of IFN-G for phase II study are 40- and 110-fold higher than that used in humans.

In the phase II IFN-G study, we discovered that ADO2 mice treated with all three doses of IFN-G exhibited either significantly lower or a trend of lower aBMD and BMC in both male and female (Figure 2 and Supplemental Table 3). Trabecular morphometry data at distal femur revealed that ADO2 mice treated with different doses of IFN-G had significantly lower BV/TV compared to the vehicle treated mice in both sexes (Figure 3 and Supplemental Table 3). In addition, mice treated with all three doses of IFN-G lowered Tb.Th by 8-13% in male and significantly lowered (16-19%) Tb.Th in female ADO2 mice. Serum biochemistry and biomarker analyses demonstrated that IFN-G treatment at all doses either significantly increased (male) or trended toward higher (female) CTX levels in ADO2 mice (Figure 4 and Supplemental Table 3). We also observed lower TRAPc5b in IFN-G treated mice compared to the vehicle group in both sexes. The CTX/TRAPc5b ratio was significantly higher in male or a trend of higher (50-75%) in female ADO2 mice as compared to the vehicle treated mice (Figure 4 and Supplemental Table 3). These data indicate that IFN-G at all doses substantially improved the osteopetrotic phenotypes in both male and female ADO2 heterozygous mice.

This study has some limitations. We did not perform quantitative histomorphometric measurements that could provide us insights for the mechanism by which IFN-gamma exerts its beneficial effect. Also, we did not investigate thoroughly the side effects of this therapy in our mouse model. Also, additional studies involving IFN-G could be undertaken to identify 1) how long the effect of IFN-G will last after the cessation of treatment, 2) whether IFN-G therapy can be as effective as continuous therapy versus treatment with on and off schedules, and 3) if any differences of treatment efficacy will exist in young versus old animals.

In conclusion, our findings indicate that while IFN-G at all doses substantially improve the osteopetrotic phenotypes in both male and female ADO2 heterozygous mice, calcitriol treatment at any dose did not improve the phenotype and at high dose further increased the bone mass. Our data do not support the continued use of calcitriol therapy in ADO2 patients and **provide support** for a clinical trial of IFN-G in these patients.

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Authors' roles

Study design: IA and MJE. Study conduct: IA, AKG, DA, RGO, AMR, and MJE. Data analysis: IA, AKG, DA, RGO, AMR, and MJE. Data interpretation: IA, AKG and MJE. Drafting manuscript: IA and MJE. Revising manuscript content: IA and MJE. Approval of final version of manuscript: IA, AKG, DA, RGO, AMR, and MJE.

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Figure Legends

Fig. 1. **Whole body aBMD and BMC measured by DXA and trabecular bone morphometry measured by micro-CT (calcitriol study).** Male ADO2 mice treated with low and medium doses of calcitriol demonstrated no differences for aBMD, BV/TV, Tb.N, Tb.Th and Tb.Sp when compared to vehicle-treated male mice. High dose calcitriol significantly increased aBMD, BV/TV and Tb.Th in male ADO2 mice as compared to the vehicle treated group. Female ADO2 mice treated with all doses of calcitriol showed similar values for aBMD, BMC, BV/TV, Tb.N, Tb.Th and Tb.Sp as compared to the vehicle group. WT-V: Wild-type vehicle, ADO2-V: ADO2 vehicle, ADO2-L: ADO2 low dose, ADO2-M: ADO2 medium dose, ADO2-H: ADO2 high dose *$p<0.05$, **$p<0.005$ compared to ADO2-V; ¶$p<0.05$ compared to WT-V

Fig. 2. **Whole body aBMD and BMC measured by DXA (Phase II IFN-G study).** Male mice treated with all doses of IFN-G and female mice treated with low dose IFN-G exhibited significantly lower aBMD as compared to the vehicle group. Both low and medium doses of IFN-G significantly reduced the whole body BMC in male ADO2 mice and there was a trend of lower (8%) BMC in high dose IFN-G treated group. The medium and high doses of IFN-G treated mice showed a trend of lower aBMD (3-4%) and all doses of IFN-G treated mice showed a trend of lower BMC (5-7%) in female ADO2 mice. WT-V: Wild-type vehicle, ADO2-V: ADO2 vehicle, ADO2-L: ADO2 low dose, ADO2-M: ADO2 medium dose, ADO2-H: ADO2 high dose *$p<0.005$, **$p<0.0005$ compared to ADO2-V; ¶$p<0.05$, ¶¶$p<0.005$ compared to WT-V

Fig. 3. A. **Micro-CT images of trabecular bone mass and micro-architecture in ADO2 mice treated with vehicle and IFN-G (Phase II IFN-G study).** Both male and female ADO2 mice treated

with vehicle exhibited twice as much bone volume (BV/TV) at distal femur compared to wild-type vehicle treated mice. ADO2 mice treated with all doses of IFN-G displayed lower bone volume and trabecular thickness as compared to the vehicle treated ADO2 mice in both male and female. B. **Trabecular bone morphometry measured by micro-CT (Phase II IFN-G study).** Male ADO2 mice treated with low and medium doses of IFN-G and female ADO2 mice treated with all doses of IFN-G demonstrated significantly lower BV/TV as compared to the vehicle treated mice. High dose IFN-G produced a trend of lower (17%) BV/TV in male mice. Female mice treated with low dose IFN-G displayed significantly lower Tb.N whereas medium and high doses of IFN-G treated mice showed a trend of lower Tb.N (8-16%) when compared to the vehicle group. Tb.Th was 8-13% lower in male ADO2 mice and significantly lower in female ADO2 mice treated with all doses of IFN-G relative to the vehicle treated mice. WT-V: Wild-type vehicle, ADO2-V: ADO2 vehicle, ADO2-L: ADO2 low dose, ADO2-M: ADO2 medium dose, ADO2-H: ADO2 high dose *$p<0.05$, **$p<0.005$ compared to ADO2-V; ¶$p<0.005$ compared to WT-V

Fig. 4. **Serum biomarkers (Phase II IFN-G study).** IFN-G treatment at all doses significantly increased (50-72%) CTX levels in male ADO2 mice. Medium dose IFN-G treated male mice exhibited a significantly lower (33%) TRAPc5b as compared to the vehicle group. In female, low dose IFN-G treatment significantly lowered the TRAPc5b (34%) amount relative to the vehicle group. The TRAPc5b/CTX ratio was significantly higher (81-133%) in male mice treated with all doses of IFN-G. Female mice treated with all doses of IFN-G also exhibited a trend of higher ratio of CTX/TRAPc5b (50-75%) compared to the vehicle treated mice. *$p<0.05$, **$p<0.005$ compared to ADO2-V; ¶$p<0.05$ compared to WT-V

Table 1. Calcium and phosphate levels in male and female ADO2 and wild-type mice treated with different doses of calcitriol and IFN-G

	WT-V	ADO2-V	ADO2-L	ADO2-M	ADO2-H
Calcitriol study					
Calcium (mg/dL)					
Male	8.55 ± 0.80	9.04 ± 0.37	8.90 ± 0.21	8.76 ± 0.33	9.18 ± 0.92
Female	8.73 ± 0.46	8.51 ± 0.60	8.80 ± 0.28	8.77 ± 0.46	9.22 ± 0.57
Phosphate (mg/dL)					
Male	6.89 ± 1.31	7.11 ± 1.01	6.79 ± 0.99	6.78 ± 0.88	7.39 ± 0.88
Female	6.67 ± 0.93	6.57 ± 0.94	6.78 ± 0.84	7.35 ± 1.28	6.98 ± 0.83
IFN-G study Phase I					
Calcium (mg/dL)					
Male	9.02 ± 0.13	8.95 ± 0.04	8.97 ± 0.12	9.16 ± 0.07	8.97 ± 0.09
Female	9.07 ± 0.09	9.18 ± 0.08	8.83 ± 0.07	9.02 ± 0.07	9.08 ± 0.07
Phosphate (mg/dL)					
Male	6.81 ± 0.15	6.82 ± 0.29	7.17 ± 0.35	7.56 ± 0.33	7.18 ± 0.45
Female	6.79 ± 0.37	6.62 ± 0.33	7.08 ± 0.38	7.80 ± 0.44	7.82 ± 0.36
IFN-G study Phase II					
Calcium (mg/dL)					
Male	9.44 ± 0.16	9.02 ± 0.13	9.56 ± 0.11	9.16 ± 0.20	8.86 ± 0.44
Female	9.31 ± 0.08	9.47 ± 0.17	9.53 ± 0.11	9.37 ± 0.15	9.59 ± 0.13
Phosphate (mg/dL)					
Male	7.57 ± 0.48	7.99 ± 0.42	8.71 ± 0.76	6.71 ± 0.22	8.97 ± 0.17
Female	8.26 ± 0.46	8.37 ± 0.49	7.03 ± 0.49	8.06 ± 0.64	6.81 ± 0.30

WT-V (Wild-type vehicle), ADO2-V (ADO2 vehicle), ADO2-L (ADO2 low dose), ADO2-M (ADO2 medium dose), ADO2-H (ADO2 high dose)
Data presented as mean ± SEM