Sustained Morphine Delivery Suppresses Bone Formation and Alters Metabolic and Circulating miRNA Profiles in Male C57BL/6J Mice

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ABSTRACT
Opioid use is detrimental to bone health, causing both indirect and direct effects on bone turnover. Although the mechanisms of these effects are not entirely clear, recent studies have linked chronic opioid use to alterations in circulating miRNAs. Here, we developed a model of opioid-induced bone loss to understand bone turnover and identify candidate miRNA-mediated regulatory mechanisms. We evaluated the effects of sustained morphine treatment on male and female C57BL/6J mice by treating with vehicle (0.9% saline) or morphine (17 mg/kg) using subcutaneous osmotic minipumps for 25 days. Morphine-treated mice had higher energy expenditure and respiratory quotient, indicating a shift toward carbohydrate metabolism. Micro-computed tomography (μCT) analysis indicated a sex difference in the bone outcome, where male mice treated with morphine had reduced trabecular bone volume fraction (Tb.BV/TV) (15%) and trabecular bone mineral density (BMD) (14%) in the distal femur compared with vehicle. Conversely, bone microarchitecture was not changed in females after morphine treatment. Histomorphometric analysis demonstrated that in males, morphine reduced bone formation rate compared with vehicle, but osteoclast parameters were not different. Furthermore, morphine reduced bone formation marker gene expression in the tibia of males (Bglap and Dmp1). Circulating miRNA profile changes were evident in males, with 14 differentially expressed miRNAs associated with morphine treatment compared with two differentially expressed miRNAs in females. In males, target analysis indicated hypoxia-inducible factor (HIF) signaling pathway was targeted by miR-223-3p and fatty acid metabolism by miR-484, -223-3p, and -328-3p. Consequently, expression of miR-223-3p targets, including Igf1r and Stat3, was lower in morphine-treated bone. In summary, we have established a model where morphine leads to a lower trabecular bone formation in males and identified potential mediating miRNAs. Understanding the sex-specific mechanisms of bone loss from opioids will be important for improving management of the adverse effects of opioids on the skeleton. © 2022 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: BONE; METABOLISM; OPIOIDS; MORPHINE; SEX DIFFERENCE; MIRNA

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Opioid use disorder has become a critical US public health concern with a staggering number of overdose deaths across the country. In addition to risk of overdose and death, there are endocrine side effects related to opioid use, and among them is increased fracture risk.1 Recently, Emeny and colleagues have estimated that opioid prescription was associated with three- to fourfold increase in fracture risk in a random sample of Medicare patients.2 Among preclinical studies, there is an observed sex difference in the effect of opioids in bone, with male animals being negatively affected.3,4 However, it should be noted that these animal models mimic diverse clinical conditions (ovariectomy versus cancer) and have not examined mechanisms of bone loss in otherwise healthy animal models.

Currently, only in vitro studies suggest opioid-induced bone changes may be due to an impairment of bone formation activity, and comprehensive in vivo analysis have not been performed. There is evidence that μ-opioid receptor (MOR) is expressed by human osteoblast-like cell line MG-635 and by human bone marrow-derived mesenchymal stem cells.6 In vitro studies suggest that opioids could directly act on bone formation by either modulating mesenchymal stem cell (MSC) fate7 or reducing mature osteoblast activity and osteocalcin synthesis.8 Despite the indices that opioids are detrimental to bone, there are limited alternative therapies for pain caused by bone fracture.9 Therefore, there is still a need to better understand the mechanisms behind opioid-induced bone alterations.

Previous studies indicate morphine tolerance–associated chronic opioid use causes dysregulation of the central and peripheral expression of microRNAs (miRNAs), small noncoding functional RNAs that modulate gene expression and various biological processes.10 In the clinical settings, Toyama and colleagues reported that patients using hydromorphone or oxycodone exhibited an upregulation of circulating miRNAs such as let-7 family and miR-339-3p, which were associated with the suppression of MOR activity.9 Moreover, morphine tolerance altered the expression of miR-93 in a bone cancer pain mouse model and affected the downstream target Smad5,10 which is important for bone homeostasis.11 However, the role of these opioid-induced miRNA changes on bone remodeling has not been systematically investigated.

Therefore, our aim was to develop a mouse model to evaluate the impact of sustained morphine exposure on bone turnover of both male and female mice and to identify candidate miRNA-mediated regulatory mechanisms that could affect bone. We initially hypothesized that chronic morphine exposure would uncouple bone turnover by reducing osteoblast and increasing osteoclast function. However, we observed no effect of morphine on osteoclasts. Briefly, we identified a sex difference in the effects of morphine on bone outcomes and circulating miRNAs. Trabecular bone loss occurred in males treated with morphine, as a consequence of impaired osteoblast function, but no changes in bone microarchitecture were observed in females. Among the enriched KEGG pathways identified, HIF signaling and fatty acid metabolism pathways were predicted to be affected by the set of miRNAs upregulated by morphine treatment in males. Our findings provide novel insight into the morphine-induced disruption of various metabolic parameters in both male and female mice, but the bone phenotype was observed exclusively in morphine-treated male mice.

Materials and Methods

Mice

Male and female C57BL/6J mice (stock #000664) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 6 weeks of age. Mice were placed in a barrier animal facility at MaineHealth Institute for Research (MHIR) on 14-hour light and 10-hour dark cycle at 22°C (standard room temperature). Mice were housed in groups of 3 or 4 per cage and they were given water and regular chow (Teklad global 18% protein diet, #2918, Envigo, Indianapolis, IN, USA) ad libitum. All mice acclimated to the MHIR animal facility for 2 weeks before the beginning of the study (day 0). All animal protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of MHIR, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–accredited facility.

Morphine delivery

Morphine sulfate salt pentahydrate powder was purchased from Sigma-Aldrich (St. Louis, MO, USA; M8777). Weight of morphine powder (in grams) used in each experiment as well as the volume of morphine solution (in μL) was logged and discarded in a locked pharmaceutical waste container. We used Alzet osmotic minipumps (Durect, Cupertino, CA, USA; model 2004; delivery rate = 0.25 μL/hr and total capacity 220 μL) to mimic a chronic exposure of morphine. The osmotic minipumps were filled under sterile conditions 40 hours before the implantation with either sterile vehicle solution (0.9% saline) or morphine solution. Morphine was administered at a dose of 17 mg/kg, based on average body weight, which was previously shown to cause bone loss but limit sedation.9 All osmotic minipumps were weighed before and after being filled to ensure the entire pump was filled and air bubbles were not present, then placed individually into sterile 0.9% saline solution at 37°C until the implantation day.

At day 0 (baseline, 8 weeks of age), mice were randomly assigned to groups and osmotic minipumps were implanted subcutaneously in all mice. Surgeries were performed in cohorts such that male and female mice were started a week apart. The entire procedure was performed in a sterile surgical field with sterile tools that were cleaned between mice. Mice were anesthetized with 2% to 3% isoflurane and kept on 2% oxygen during the whole procedure. After shaving and sterilizing with betadine, a cutaneous dorsal incision was made perpendicular to the spine, approximately 0.5 inches caudal to the base of the neck. A hemostat was used to clear space for the osmotic minipump caudal to the incision, such that the inserted minipump was located closer to the hind quarters and would not interfere with healing of the incision. The incision was closed using wound clips, and all mice received 1 mg/kg sc meloxicam (Patterson Veterinary, Loveland, CO, USA) b.i.d. for 1 day after the surgery. Mice were weighed before and after the osmotic minipump implantation, and the latter was considered the baseline weight measure. Mice were examined for signs of pain or distress twice a day during 4 consecutive days after the surgery and the wound was examined for signs of inflammation. We euthanized one male from the vehicle group before the endpoint of the study because it exhibited impaired wound healing and signs of distress. We finished this 25-day experiment with a total of 12 male mice in the vehicle group, 14 male mice in the morphine group, 11 female mice in the vehicle group, and 11 female mice in the morphine group. Euthanasia was performed after 25 days of
morphine treatment (12 weeks of age) using isoflurane anesthesia followed by decapitation (except where noted below).

Metabolic cage system

A total of 8 male and female mice from each treatment group (vehicle and morphine groups) were placed individually into metabolic cages for 5 days twice during the entire experiment (total of 10 days: 5 days on week 2 and 5 days on week 4) to assess metabolic and behavioral changes using the Promethion metabolic cage system (Sable Systems International, North Las Vegas, NV, USA), located in the Physiology Core at MMCRI. We are limited to 16 cages, therefore the number for metabolic cage data is only 8 per group (male and female mice were tested during separate weeks). Data acquisition and instrument control were performed using Meta Screen version 1.7.2.3, and the raw data obtained were processed with ExpeData version 1.5.4 (Sable Systems International) using an analysis script detailing all aspects of data transformation. Summary 24-hour metabolic and behavioral assessment are presented. On occasion, technical problems occur with specific components of the cages (ie, water bottle weight measurements absent but activity data normal). Inaccurate data were excluded when such problems were identified, which resulted in a smaller number for some measurements.

Circulating morphine and morphine metabolite measurement

Serum concentrations of morphine and morphine metabolites, morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G), were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis, based on the method of Clavijo and colleagues. Morphine, M-3-G, and M-6-G were extracted from serum via protein precipitation with acetonitrile. Separation was accomplished using a Phenomenex Synergi Hydro-RP analytical column (2.0 mm, 4 μm). Mobile phase consisted of 0.1% formic acid in purified water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.5 mL/min and heated to 30°C. Gradient elution was employed, with initial conditions 97% A and 3% B. Solvent composition was held at the initial conditions for 1.5 minutes and then was ramped over the next 2.0 minutes to 25% B. Morphine and its metabolites were detected via an Agilent (Waldbronn, Germany) 6460 triple quadrupole mass spectrometer operated in positive ion MRM mode. The following transitions were monitored: morphine (286.1 → 152.0), M-3-G and M-6-G (462.5 → 286.1). Circulating morphine and morphine metabolites levels were determined in an aliquot of serum after sustained treatment of 25 days (first experiment) and 12 days (second experiment).

Dual-energy X-ray absorptiometry (DXA)

All mice were weighed before DXA measurement. Areal bone mineral content (aBMC), areal bone mineral density (aBMD), lean mass, and fat mass measurements were performed on each mouse at baseline by a PIXImus dual-energy X-ray densitometer (GE Lunar, GE Healthcare, Madison, WI, USA). The PIXImus was calibrated daily with a mouse phantom provided by the manufacturer. Mice were placed ventral side down with each limb and tail positioned away from the body. Full-body scans were obtained, and the head was excluded from analysis because of concentrated mineral content in the skull and teeth. X-ray absorptiometry data were processed and analyzed with Lunar PIXImus 2 (version 2.1) software. DXA measurements were repeated on mice that underwent metabolic cage testing at weeks 2 and 4.

Micro-computed tomography (µCT)

A high-resolution desktop micro-tomographic imaging system (µCT40, Scanco Medical AG, Brüttisellen, Switzerland) was used to assess trabecular bone architecture in the distal femoral metaphysis and L5 vertebral body and cortical bone morphology of the femoral mid-diaphysis of male and female, vehicle and morphine-treated mice, ex vivo, after 25 days of treatment. Final femoral sample numbers include, in males, n = 12 vehicle and n = 14 morphine; and in females, n = 11 vehicle and n = 11 morphine. Final L5 vertebral sample numbers include, in males, n = 10 vehicle and n = 14 morphine; and in females, n = 11 vehicle and n = 10 morphine. L5 samples that were damaged during bone collection were excluded from the analysis (two male vehicle samples and one female morphine sample). Scans were acquired using a 10 μm^3 isotropic voxel size, 70 kVP, 114 μA, 200 ms integration time, and were subjected to Gaussian filtration and segmentation. Image acquisition and analysis protocols adhered to guidelines for the assessment of rodent bones by µCT. In the femur, trabecular bone microarchitecture was evaluated in a 1500 μm (150 transverse slices) region beginning 200 μm superior to the peak of the growth plate and extending proximally. In the L5 vertebral body, trabecular bone was evaluated in a region beginning 100 μm inferior to the cranial endplate and extending to 100 μm superior to the caudal endplate. The trabecular bone regions were identified by manually contouring the endocortical region of the bone. Thresholds of 335 mgHA/cm^3 and 385 mgHA/cm^3 were used to segment bone from soft tissue in the femur and L5 vertebrae, respectively. The following architectural parameters were measured using the Scanco Trabecular Bone Morphometry evaluation script: trabecular bone volume fraction (Tb.BV/Tv, %), trabecular bone mineral density (Tb.BMD, mgHA/cm^3), specific bone surface (BS/BV, mm^2/mm^3), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm^-1), trabecular separation (Tb.Sp, mm), and connectivity density (Conn.D, 1/mm^3). Cortical bone was assessed in 50 transverse µCT slices (500 μm long region) at the femoral mid-diaphysis, and the region included the entire outermost edge of the cortex. Cortical bone was segmented using a fixed threshold of 708 mgHA/cm^3. The following variables were computed: total cross-sectional area (bone + medullary area) (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), medullary area (Ma.Ar, mm^2), bone area fraction (Ct.Ar/Tt.Ar, %), cortical tissue mineral density (Ct.TMD, mgHA/cm^3), and cortical thickness (Ct.Th, mm). Cortical bone images were taken of the mouse using the median total area value within each group.

Histomorphometric bone analysis

Bone histomorphometric analysis was performed on the left femur of male treated with vehicle solution and with morphine solution for 25 days. Calcein solution (20 mg/kg; Sigma) and Alizarin solution (40 mg/kg) were injected at 8 days and 2 days before animal euthanasia, respectively. The femur was dissected and formalin-fixed (10%) for 48 hours before transferred to 70% ethanol solution. Fixed, nondecalcified samples were dehydrated using graded ethanol solutions and subsequently infiltrated and embedded in methylmethacrylate. Longitudinal sections (5 μM) were cut using a microtome (RM2255, Leica,
Wetzlar, Germany) and stained with Goldner’s Trichrome for measurements of bone microarchitecture and cellular parameters. Dynamic bone parameters were evaluated on unstained sections by measuring the extent and the distance between double labels using the Osteoneasure analyzing system (OsteoMetrics Inc., Decatur, GA, USA). Measurements were made in the same position for each sample at 3600 μm² area in the femur (200–250 μm below growth plate). Quantification of bone parameters was done in a blinded manner. The structural, dynamic, and cellular parameters were evaluated using standardized guidelines.19 The histomorphometric bone measures done are as follows: bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm), trabecular separation (Tb.Sp, μm), osteoid volume (OV/BV, %), osteoid thickness (O.Th, μm), absolute number of osteocyte (N.Ot), number of osteocytes/bone area (Ot/B.Ar, n/mm²), osteoblast surface (Ob.S/B.S, %), osteoblast number per bone perimeter (N.Ob/B.Pm, mm), mineralizing surface per bone surface (MS/BS, %), mineral apposition rate (MAR, μm/d), bone formation rate per bone surface (BFR/BS, μm²/μm²/d), osteoclast surface per bone surface (Oc.S/BS, %), osteoclast number per bone perimeter (N.Oc/B.Pm, mm), and eroded surface (ES/BS, %).

Real-time PCR
Tibias were collected from 12-week-old male and female mice from vehicle and morphine groups for RNA extraction under liquid nitrogen conditions (n = 7–14). For the opioid receptor expression analysis, we used a set of nontreated 8-week-old quantiﬁcation.15 System thermal cycler and detection system. TATA binding protein 1 (Tbp1) was used as an internal standard control gene for all expression analysis, we used a set of nontreated 8-week-old mice. cDNA was generated using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. mRNA expression analysis was carried out in duplicate using an iQ SYBR Green Supermix with a Bio-Rad Laboratories (Hercules, CA, USA) CFX Connect Real-time System thermal cycler and detection system. TATA binding protein 1 (Tbp1) was used as an internal standard control gene for all quantification.16 Primers used were from Integrated DNA Technologies (IDT, Coralville, IA, USA).17–19 Primer Design (Southampton, UK), or Qiagen (Germantown, MD, USA). All primer sequences (if provided) are listed in Table S1. Total RNA integrity of the samples from the 25-day treatment experiment was evaluated by running a denaturing 1% agarose gel stained with ethidium bromide (EtBr). The number of samples included in the statistical analysis varied from 7 to 14 per group, depending on the quality of the RNA. For the opioid receptor expression analysis, the number of samples included varied from 6 to 8.

Serum turnover markers
Serum P1NP and CTx-1 were measured as previously described and according to the manufacturer instructions.20

microRNA array analysis—12-day experiment
A second cohort of male and female mice were implanted subcutaneously with osmotic minipumps filled with vehicle or morphine solution as described earlier. DXA analyses also were performed during this experiment. Mice were euthanized by day 12 using isoflurane. For each animal, whole blood was collected at death in a 1.5 mL tube using cardiac puncture, allowed to clot for 30 minutes at room temperature, and then placed on ice. All blood samples were centrifuged for 10 minutes at 500 relative centrifugal force (RCF). At least 200 μL serum was removed from the top translucent phase and stored at −80°C (n = 3–5 male and n = 3–4 female). All mice were 10 weeks of age at the endpoint. Total RNA was isolated from 200 μL mouse serum following a standardized protocol21 using the miRNeasy Serum Plasma kit with ce-miR-39 spike-in (Qiagen), QIAcube (Qiagen) automation, and eluted with 14 μL of nuclease-free water.

Global circulating miRNA screen
Exactly 8 μL of isolated RNA was prepared for Affymetrix GeneChip miRNA v4 microarrays (Thermo Fisher Scientific, Waltham, MA, USA), allowed to hybridize for 42 hours, and data processed as described.22 The R script for this normalization is included as a supplement. Differential expression significance was assessed by one-way ANOVA. The data have been deposited into the GEO repository (GSE197198).

Statistical analysis
GraphPad (La Jolla, CA, USA) Prism 9 XML Project software was used to perform statistical tests. Data are presented as mean ± standard deviation (SD). Outliers were defined as data points >3 standard deviations from the mean and were excluded from analysis. Student’s t test or two-way ANOVA was performed and Holm-Sidak post hoc multiple comparison test was performed where appropriate (after a significant interaction effect). α ≤ 0.05 was considered statistically significant. Heat-map values and principal component analysis were generated in Spotfire v2 (Tibco, Palo Alto, CA, USA). All data were imported into Adobe Illustrator CC for final figure creation. Volcano plot analysis was performed to identify miRNA with large-fold changes (threshold of ≥2-fold change) that were also statistically significant (p < 0.01). Diana miRPath v3.0 software was used to search for predicted affected experimentally validated (Tarbase V.7) and nonexperimentally validated (Target Scan) enriched KEGG pathways23 at p values <0.05, with the following settings: pathways union, false discovery rate (FDR) correction box checked, and conservative stats box unchecked. For this, we evaluated each miRNA separately to identify the potential KEGG pathways affected by each one of them.

Results
Male mice exhibited reduced femoral trabecular bone after sustained morphine delivery
We first wanted to investigate whether sustained morphine delivery caused changes in bone. Areal bone mineral density was evaluated at three time points during the study (baseline, day 7, and day 21). After 7 days of the osmotic minipump implantation, male and female mice exhibited a drop in aBMD, regardless of treatment groups. However, sustained morphine delivery did not worsen this initial aBMD loss, which may have been caused by the surgical procedure (Fig. 51C). Morphine treatment affected femoral trabecular bone in male mice, which exhibited reduced Tb.BV/TV (15%, p = 0.035) and Tb.BMD (14%, p = 0.015) compared with the vehicle group (Fig. 1A–C). We observed no changes related to morphine treatment in any other bone parameters evaluated, such as Tb.BS/BV, Conn.D, Tb.N, Tb.Th, and Tb.Sp (p > 0.05) (Fig. 1D–F). Unlike males, however, sustained morphine delivery did not alter any of the trabecular bone parameters evaluated in female mice, which were
(Figure legend continues on next page.)
similar between treatment groups ($p > 0.05$) (Fig. 1A–H). Because of these differences, circulating levels of morphine and metabolites were measured to ensure that the drug solution was still being delivered by the osmotic minipumps after 25 days. We detected morphine and morphine-3-glucuronide (M-3-G) in the serum of morphine-treated male and female mice at the endpoint ($p < 0.0001$), whereas morphine-6-glucuronide (M-6-G) levels were undetectable (Table S2). We also found that morphine levels were similar between sexes, whereas females have a higher circulating level of M-3-G compared with males (Fig. S1A, B).

On the other hand, unlike the effects found in the trabecular compartment, our μCT findings revealed that morphine treatment had no effect on cortical bone (Fig. 2), which is consistent with aBMD data that usually reflects alterations in cortical microarchitecture. We observed that there were no changes in T.T.Ar, M.A.R, and C.T.Ar after morphine exposure in either in males or females ($p > 0.05$) (Fig. 2B–D). Both morphine-treated male and female mice also exhibited similar C.T.Ar/T.T.Ar, C.T.Th, and C.T.TMD compared with vehicle groups ($p > 0.05$) (Fig. 2E–G).

In contrast to femoral trabecular bone results, morphine treatment had no significant impact on L5 vertebral body microarchitectural parameters (Fig. 3A–H), although vertebral BV/TV and BMD tended to be lower in male mice treated with morphine compared with vehicle.

Morphine caused reduced osteoblast mineralization activity

We next performed histomorphometric analysis in the femur to better understand morphine-induced changes in bone turnover in male mice. This analysis also confirmed that chronic morphine was associated with decreased femoral BV/TV and Tb.Th in males ($p < 0.0001$) (Table 1). Morphine-treated male mice also exhibited reduced bone area (B.Ar) compared with vehicle group ($p < 0.0001$). Unexpectedly, we found that the number of osteoblasts (Ob.S/BS) and the number of osteoclasts (Oc.S/BS) per bone surface were similar between vehicle and morphine groups after 25 days of treatment ($p > 0.05$). However, morphine exposure significantly decreased BFR/BS ($p = 0.033$) (Table 1). Moreover, we also observed a trend toward reduced MAR ($p = 0.086$), MS/BS ($p = 0.055$), and decreased osteoclast activity (eroded surface, ES/BS) ($p = 0.077$) in male mice. However, these latter parameters did not reach statistical significance (Table 1).

In addition, we found reduced N.Ot but not N.Ot corrected by bone area (Table 1). We also evaluated the expression of osteoblast/osteocytes and osteoclast markers in the whole tibia from male and female mice (Fig. 4A, B). As expected, the expression data corroborated our histomorphometric results. We found a reduced expression of Bglap, Dmp1, and Fgf23 in the whole tibia of morphine-treated male compared with vehicle-treated male mice ($p < 0.001$), suggesting that morphine has a major impact in the function of mature osteoblast lineage cells (Fig. 4A). Our findings also demonstrated a reduction in the expression of Ctsk ($p < 0.05$) (Fig. 4A). On the other hand, we found that sustained morphine treatment had no effect on Rankl/Opg system, Tnfsf11b (Opg) expression, Tnfsf11 (Rankl) expression, and Rankl/Opg ratio were similar between treatment groups ($p > 0.05$) (Fig. 4A). Despite the absence of altered bone microarchitecture in females, we also examined gene marker expression. We found significant decreases in Runx2 and Dmp1 in females treated with morphine (Fig. 4B). Serum P1NP was also significantly suppressed in females but not males (Fig. 4C, D), whereas CtX-1 only tended to be lower in male mice (Fig. 4C). Collectively, these findings suggest altered bone formation is the predominant mediator of bone loss in mice treated with morphine and that female mice may not be completely protected.

We next wanted to confirm the magnitude of the opioid receptors (MOR, DOR, and KOR) expression in whole tibia of male and female mice because others have shown MOR is expressed by osteoblast-like cells. However, expression of the opioid receptors was exceptionally low to absent, and they were significantly lower compared with brain expression of these receptors ($p < 0.0001$) (Fig. S2), which suggests that morphine likely has an indirect effect on the bone.

Morphine treatment outcomes in body weight and composition

Differences in body weight and composition between male and female were observed, as expected (Table S3). At the baseline, treatment groups started with similar body weight and body composition evaluated by two-dimensional DXA analysis. Conversely, after 21 days of sustained morphine exposure, we found a significant reduction in the % of fat mass and adiposity index (Table S3). However, we observed no sex by treatment interaction effects, suggesting that morphine influenced body weight and composition similarly in both sexes.

Morphine treatment had no major effect on motor activity

We performed a 2-way ANOVA analysis to better understand the effects of sustained morphine exposure in metabolic and motor activity over time (from weeks 2 to 4). Overall, we found that sustained morphine exposure had no major impact in motor activity in morphine-treated mice. In males, we observed that sustained morphine treatment led to reduced X beam breaks within the cage ($p < 0.05$) (Table S4), but this was not enough to influence the distance walked or time spent walking in the metabolic cages. Furthermore, morphine did not influence time or speed on running wheels in either sex of mice. Over time, there was
an increase in Z beam breaks movements and wheel speed \( (p < 0.05) \), with no differences between treatment groups, which was likely related to surgery recovery. In females, morphine caused a reduction in the percentage of time spent staying still compared with the vehicle group \( (p < 0.05) \) (Table S4). From weeks 2 to 4, in general, females became less active, which was observed by a reduction in X and Y beam movements, cage walking meters, and percentage of time spent walking and an increase in the percentage of time sleeping \( (p < 0.05) \). However, this behavior was similar between groups with no statistical difference (Table S4). Moreover, there was no treatment by time point interactions effect for any of the motor activity parameters assessed \( (p > 0.05) \) (Table S4). Our findings suggest that sustained morphine exposure had only a minimal impact on motor activity.

Sustained morphine treatment led to changes in energy expenditure and fuel utilization

In males, sustained morphine exposure increased energy expenditure (EE), \( \text{CO}_2 \) expelled, respiratory quotient (RQ), resting respiratory quotient (RRQ), and active respiratory quotient (ARQ)
Fig. 3. Vertebral L₅ trabecular bone compartment was preserved in male and female mice after chronic morphine exposure. (A) Representative μCT images from L₅ vertebrae. (B–H) L₅ vertebrae μCT from mice treated with vehicle (VEH, male = blue; female = green) and morphine solution (MOR, male = orange; female = purple) for 25 days. Parameters include trabecular bone volume (BV/TV), trabecular bone mineral density (Tb.BMD), trabecular specific bone surface (Tb.BS/BV), trabecular connectivity (Conn.D), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Number of mice/group: vehicle (male = 10; female = 11) and morphine (male = 14; female = 10). Boxplots demonstrate median, minimum, and maximum values. Symbol of plus sign corresponds the mean. Main effect p values of two-way ANOVA are denoted below each graph. Post hoc tests were only performed after a significant interaction effect, and those p values are denoted above the brackets.
resting energy expenditure (REE) was also increased in morphine-treated male mice compared with vehicle group \((p = 0.0001)\), although there was a reduction of REE values over time (from weeks 2 to 4) \((p = 0.002)\) (Table S5). However, we found no treatment by time point interactions effect for any of the metabolic parameters assessed (Table S5). In addition, while an increase in water consumption over time was observed, food intake of male mice was not significantly affected by sustained morphine treatment, time of morphine exposure, or interaction effect between variables (Table S5). In females, we also observed an increase in EE, CO\(_2\) expelled, \(O_2\) consumed, REE, active energy expenditure (AEE), and ARQ related to chronic morphine treatment. We found that \(CO_2\) expelled and AEE also increased over time (from weeks 2 to 4), but there were no treatment by time point interaction effects for these parameters (Table S5). Because we found a significant treatment by time point interaction for RQ data in females, we performed Holm-Sidak post hoc multiple comparison test, which demonstrated that RQ was higher in morphine-treated female mice compared with vehicle mice at both time points \((p < 0.001)\), but from weeks 2 to 4, there was no significant changes in RQ related to morphine treatment \((p = 0.074)\) (Table S5). Together, these findings seem to indicate that a greater length of continuous morphine exposure (4 weeks) had no further effect on energy expenditure and fuel utilization, and changes in metabolism appear to begin at an early stage of morphine treatment (2 weeks).

Sustained morphine exposure induced sex-difference changes in circulating miRNA profile

To further understand the potential molecular mechanisms that might be involved in the sustained morphine-induced bone phenotype, serum was collected from male and female C57BL/6J mice treated with morphine for 12 days to perform miRNA array analysis (Fig. 5). Circulating levels of morphine and its metabolites (M-3-G and M-6-G) were also measured to confirm that the osmotic minipumps were delivering the drug (Table S2). At this short-term experiment, after 12 days, females exhibited a trend toward a higher concentration of morphine \((p = 0.051)\) and M-3-G levels \((p = 0.066)\) compared with males (Fig. S1E). Interestingly, we found a sex difference in the miRNA profile associated with morphine exposure. Of 1908 mature miRNAs evaluated, morphine induced changes in 260 miRNAs. Principal component analysis (PCA) demonstrated that morphine treatment seems to induce males to have an miRNA profile close to female mice (Fig. S1F). The heat map illustrates differences in miRNA expression between vehicle-and morphine-treated male mice after 12 days of morphine treatment, which overall had a suppression of miRNA from morphine (Fig. 5A). We also identified that in females only 2 miRNAs were significantly and differentially expressed miRNAs, whereas in males there were 14 differentially expressed miRNAs with morphine that reached a threshold of two-fold change and \(p < 0.01\) (Fig. 5B).

Fatty acid metabolism and HIF signaling pathways are targeted by sustained morphine treatment

Of those 14 differentially expressed miRNAs in morphine-treated male mice, 4 miRNAs were upregulated and 10 miRNAs were downregulated \((p < 0.05)\) (Table 2). Many of these miRNAs have a previously known connection to bone (summarized in Table 2).

Most of the upregulated miRNAs found (miR-484, -223-3p, and -328-3p) were associated with 4 to 11 experimentally and non-experimentally validated enriched KEGG pathways \((p < 0.05)\) (Table 3). In general, these miRNAs were found

| Table 1. Histomorphometric Analysis of the Distal Region of the Femur from Vehicle- and Morphine-Treated Male Mice |
|---------------------------------------------------------------|
| Histomorphometric variables | Vehicle group \((n = 7)\) | Morphine group \((n = 8)\) | \(p\) Value |
|-----------------------------|--------------------------|--------------------------|------------|
| B.Ar (mm\(^2\))             | 0.13 ± 0.01              | 0.09 ± 0.01              | <0.0001    |
| BV/TV (%)                   | 13.3 ± 1.2               | 9.8 ± 1.1                | <0.0001    |
| Tb.Th (mm)                  | 41.1 ± 2.9               | 30.5 ± 4.2               | <0.0001    |
| Tb.Sp (mm)                  | 269.2 ± 25.3             | 279.2 ± 10.8             | 0.326      |
| OV/BV (%)                   | 0.010 ± 0.008            | 0.007 ± 0.005            | 0.395      |
| O.Th (μm)                   | 0.50 ± 0.14              | 0.41 ± 0.14              | 0.259      |
| N.Ot                        | 65.6 ± 8.3               | 46.1 ± 7.6               | <0.001     |
| Ot/B.Ar (n/mm\(^2\))        | 512.7 ± 37.6             | 492.2 ± 52.3             | 0.406      |
| Ob.S/BS (%)                 | 9.3 ± 2.2                | 8.3 ± 1.2                | 0.286      |
| N.Ob/B.Pm (mm)              | 13.5 ± 3.6               | 12.0 ± 2.2               | 0.328      |
| BFR/BS (μm/3 µm\(^2\)/d)   | 0.0010 ± 0.0004          | 0.0006 ± 0.0004          | 0.033      |
| MAR (µm/d)                  | 0.10 ± 0.04              | 0.07 ± 0.03              | 0.086      |
| MS/BS (%)                   | 1.1 ± 0.3                | 0.8 ± 0.3                | 0.055      |
| Oc.S/BS (%)                 | 3.3 ± 1.2                | 3.6 ± 1.4                | 0.658      |
| N.Oc/B.Pm (mm)              | 2.1 ± 0.8                | 2.4 ± 0.8                | 0.488      |
| ES/BS (%)                   | 1.1 ± 0.3                | 0.8 ± 0.3                | 0.077      |

\(B.Ar = \)bone area; \(BV/TV = \)bone volume; \(Tb.Th = \)trabecular thickness; \(Tb.Sp = \)trabecular separation; \(OV/BV = \)osteoid volume; \(O.Th = \)osteoid thickness; \(N.Ot = \)absolute number of osteocytes; \(Ot/B.Ar = \)number of osteocytes/bone area; \(Ob.S/BS = \)osteoblast surface; \(N.Ob/B.Pm = \)osteoblast number per bone perimeter; \(BFR/BS = \)bone formation rate per bone surface; \(MAR = \)mineral apposition rate; \(MS/BS = \)mineral apposition rate per bone surface; \(Oc.S/BS = \)osteoclast surface per bone surface; \(N.Oc/B.Pm = \)osteoclast number per bone perimeter; \(ES/BS = \)eroded surface.

Data presented as mean ± standard deviation (SD).
to target experimentally validated pathways related to energy metabolism, especially fatty acid metabolism (Table 3). Interestingly, we also found that miR-223-3p affected HIF-1 signaling pathway, which has been reported to influence both osteoblast and osteoclast cells. In addition, we observed that miR-484 and miR-328-3p targeted non-experimentally validated pathways related to morphine and nicotine addiction (Table 3). We did not find any experimentally or non-experimentally validated results for miR-3107-3p (aka, miR486b-5p) when performing target pathway analysis.

The downregulated miRNAs were associated with 1 to 21 experimentally and non-experimentally validated enriched KEGG pathways (Table 4). (TBL 4) Different from the findings related to the upregulated miRNAs, we observed that over half of the downregulated miRNAs (miR-200b-3p, -351-5p, -203-3p, -200c-3p, -125b-5p, and -200b-5p) have experimentally validated miRNA/mRNA targets (Tarbase V.7) (Table 4). Mostly, the 11 downregulated miRNAs found affected experimentally and non-experimentally validated pathways related to protein metabolism and metabolic pathways. Interestingly, miR-125-5p was found to target 21 different experimentally validated pathways, which was the most of any of our miRNAs assessed (Tarbase V.7) (Table 4).

In female mice, we found the expression of miR-1982-5p and -3090-5p were upregulated after morphine exposure. There were no related experimentally validated enriched pathways found (Tarbase V.7). However, when searching for the non-experimentally validated enriched pathways (Target Scan), we found that miR-3090-5p was mostly associated with fatty acid metabolism (Table S6).

To determine if the miRNA changes could be responsible for bone effects in male mice, we tested if any of the downregulated genes (Fig. 4A) were targets of the upregulated miRNAs (Table 2) and found that none were experimentally validated or predicted targets of the upregulated genes from male mice. We next examined expression of a subset of targets of the upregulated miRNAs from male mice that comprised the enriched HIF-1α and fatty acid metabolism pathways. Some targets of miR-223-3p, including insulin-like growth factor 1 receptor (Igf1r), eukaryotic translation initiation factor 4E family member 2 (Eif4e2), and signal transducer and activator of transcription (Stat3), were significantly suppressed by morphine treatment (Fig. 6). Similarly, expression of the miR-484 target Oxsm, which encodes the protein 3-0xacyl-ACP synthase, was also significantly lower in the morphine-treated mouse bone (Fig. 6). Further studies will be required to determine if these and/or other miRNA/target gene changes mediate morphine effects on bone.

**Discussion**

Sustained opioid use has previously been associated with deleterious effects on the skeleton and a higher fracture risk. In this study, our aim was to develop a mouse model of opioid-induced bone loss to study the impact of chronic morphine exposure on bone turnover and to identify potential miRNA-mediated regulatory mechanisms that contribute to the effects of morphine on bone tissue. To our knowledge, we are the first to evaluate the effects of sustained morphine treatment on the skeleton, body composition, metabolic and motor activity, and circulating miRNA in male and female C57BL/6J mice. Our initial hypothesis was that chronic morphine exposure would lead to bone loss by uncoupling bone turnover through suppression of bone formation and increasing bone resorption. However, the resulting data did not indicate changes in osteoclast activity during morphine treatment. Rather, the present study found that sustained morphine exposure for 25 days produced a reduction in osteoblast activity and decreased expression of bone-forming genes, which led to a lower trabecular bone in male mice. Moreover, morphine treatment modulated the expression of miRNAs, more so in males than in females, and some of the targets of elevated circulating miRNAs were significantly downregulated in bone.

Sex differences in bone outcomes between males and females are noticeable among studies investigating the effects of opioid on bone in humans. Opiod-induced bone loss has been consistently reported in men whereas the
effects of opioids on BMD in women are less clear.\(^{41,44}\) Some studies show that long-term opioid treatment (>5 years) has no significant effect on BMD changes over time in women.\(^{41,44}\) However, opioid treatment appears to be more harmful to bone compared with other analgesics evaluated (acetaminophen and nonsteroidal anti-inflammatory drugs)\(^ {44}\) and remains associated with increased fracture risk in women.\(^ {2,45}\) Despite the similar circulating levels of morphine, in males and females, our findings revealed a clear lack of morphine effects on the skeleton of females compared with males, which corroborates with the existing data.\(^ {3,4}\) An apparent explanation for this is that opioid use is described to cause reduction in circulating testosterone levels.\(^ {40}\) However, bone phenotype observed in morphine-treated male mice is not as dramatic as that of orchiectomized animal model. The reduced testosterone levels obtained in an orchiectomized model leads not only to a dramatic decrease in trabecular bone volume but also impaired cortical thickness.\(^ {46}\) Additionally, bone loss observed in the orchiectomized animal model is associated with an increased osteoclast activity.\(^ {46}\) Although microarchitectural parameters were unchanged in females, we did find reduced gene expression of bone formation markers and reduced serum P1NP, suggesting a longer treatment, higher dose, or differential treatment context (ie, with cancer) of morphine may alter bone parameters.

We found that osteoblast cell number was not affected by chronic morphine exposure, but morphine had a deleterious effect on bone formation rate, also appearing to have a trend toward a reduced mineralized surface and mineral apposition rate in the femur region. These data were supported by reduced gene expression of osteoblast osteocyte markers (Bglap, Dmp1, Fgf23) in males exposed to the drug. This is consistent with the very limited evidence demonstrating that opioids have a direct impact on osteoblasts.\(^ {35}\) Ultimately, whether direct effects are important in our findings is unclear, since opioid receptor expression in bone is very low to absent. In contrast, we did not observe any effects of opioids on bone resorption. We found no changes in the numbers of osteoclasts with chronic morphine exposure, different from what we were expecting. In fact, our data suggest that chronic morphine treatment may have a suppressive effect on bone resorption function with reduced expression of Ctsk in the whole tibia. Alonso-Pérez and colleagues\(^ {47}\) described that morphine can signal through a toll-like receptor 4 (TLR4)/myeloid differentiation protein 2 (MD-2) complex, which is expressed by osteoblasts, and indirectly modulates osteoclast functionality.\(^ {47}\) However, our data have not demonstrated a clear change in osteoclast activity. Alternately, the source of Ctsk could be osteocytes or periosteal stem cells and reduction in Ctsk in morphine-treated mice could be a reflection of the reduction in other osteocyte markers.\(^ {48,49}\) Future studies examining osteoblast maturity and osteocyte morphology in vivo, and whether circulating FGF-23 and phosphate metabolism are altered with morphine treatment, may be warranted given the decreases in late osteoblast and osteocyte markers.

Our focus on the miRNAs as one of the potential mechanisms contributing to morphine effects in the skeleton also revealed a sex difference in miRNA profile related to morphine

Fig. 5. Sustained morphine exposure induced sex-difference changes in circulating miRNA profile. (A) Heat map of the miRNA profile expressed after 12 days of morphine exposure in the vehicle- versus morphine-treated male mice represented using log2-fold change values. *p < 0.05. (B) Volcano-plot analysis of the differentially expressed miRNAs in vehicle- versus morphine-treated male and female mice, considering the threshold of ≥2-fold change and p < 0.01 as statistically significant.
Table 2. Upregulated and Downregulated List of Significantly Differently Expressed miRNA in Males After Morphine Exposure

| Circulating miRNAs (male mice) | log2fold | p Value | Known association with bone? | Reference |
|-------------------------------|----------|---------|-------------------------------|-----------|
| Upregulated:                  |          |         |                               |           |
| miR-484                       | 2.810    | 0.003   | Directly related to bone mineral density (BMD) in femur but negatively associated with that of iliac in postmenopausal women with normal to osteoporotic bone. | Gautvik et al.\(^{24}\) |
| miR-223-3p                    | 1.667    | 0.001   | Upregulated in individuals with a WNT7 mutation. | Makitie et al.\(^{25}\) |
| miR-328-3p                    | 1.302    | 0.008   | Together with miR-484, miR-328-3p is directly related to bone mineral density (BMD) in femur but negatively associated with that of iliac in postmenopausal women with normal to osteoporotic bone. | Gautvik et al.\(^{24}\) |
| miR-486b-5p (formerly miR-3107-5p) | 1.375    | 0.003   | Highly expressed in exosomes from cultured murine bone marrow-derived MSCs. | Luther et al.\(^{26}\) |
| Downregulated:                |          |         |                               |           |
| miR-200b-3p                   | −5.294   | 0.004   | H19/miR200b-3p/ZEB1 axis contributed to hepatocellular carcinoma bone metastasis. H19 enhances cell migration and invasion by upregulating zinc finger E-box binding homeobox 1 (ZEB1) by sequestration of miR-200b-3p. | Huang et al.\(^{27}\) |
| miR-28a-3p                    | −4.311   | 0.009   | None known.                   | Bai et al.,\(^{28}\) Pan et al.,\(^{29}\) and Legrand et al.\(^{30}\) |
| miR-182-5p                    | −3.670   | 0.002   | When overexpressed in human bone marrow mesenchymal stem cells, miR-182-5p inhibits chondrogenesis, decreases expression levels of SOX9 and COL2A1, and increases expression levels of COL1A1 and COL10A1. miR-182-5p upregulation was found in patients with fibrous dysplasia, demonstrating a potential involvement on the dysregulation of gene expression in bone. On the other hand, downregulation of miR-182-5p seems to promote osteoblast proliferation and differentiation in osteoporotic rats through Rap1/MAPK signaling pathway activation by upregulating ADCY6. | Nishi et al.\(^{31}\) |
| miR-6769b-5p                  | −3.372   | 0.009   | Overexpressed by bone marrow-derived macrophages leading to a phenotypic switch to fibroblastic macrophages with increased MMP9 expression and reduced ATF4 and inflammatory cytokine production. | Huang et al.\(^{32}\) |
| miR-351-5p                    | −2.751   | 0.002   | Involved in the regulation of Disheveled 2 (Dvl-2), a key mediator of the Wnt/β-catenin signaling pathway. | Niu et al.\(^{33}\) and Tang et al.\(^{34}\) |
| miR-203-3p                    | −2.597   | 0.006   | Downregulation of IncRNA X-inactive specific transcript (XIST) promotes osteoblast proliferation and differentiation and also inhibits apoptosis by regulating miR-203-3p/zinc finger protein multitype 2 (ZFPMT2) axis. miR-203-3p also inhibits osteogenesis in jaw bones of diabetic rats by negatively targeting Smad1 and Runx2. | — |
| miR-7002-5p                   | −2.495   | 0.007   | Involved in the regulation of BMP4/Smad pathway during skeletal fluorosis. Moreover, miR-200c-3p/smadmin7 axis is regulated by zingerone, 4-(4-hydroxy-3-methoxyphenyl)-2-butane (ZG) promoting expression of osteoblast markers (ALP, OC, OSX, and RUNX2) in human bone mesenchymal stem cells. | Jang et al.\(^{35}\) and Song et al.\(^{36}\) |
| miR-125b-5p                   | −2.341   | 0.008   | Upregulated in osteoblast and osteoclast cells, serum, and tissue of osteoporotic patients, with a sex-dependent expression. In a separate study, it was downregulated in a group of osteoporotic subjects. | Kelch et al.\(^{37}\) and Chen et al.\(^{38}\) |
| miR-200b-5p                   | −2.263   | 0.005   | None known.                   | —         |

Treatment, with more prominent morphine-induced changes in miRNA profile in males than in females. Notably, in contrast to previous data, we have not found the expression of any miRNA (eg, let-7) known to be associated with MOR activity.\(^{37}\) The experimentally validated enriched KEGG pathways, targeted by the miRNAs, were not directly associated with morphine or opioid-related pathways. However, miR-484 and -328-3p were predicted to affect non-experimentally validated enriched KEGG pathways related to morphine addiction. Moreover, the expression of these two miRNAs (miR-328-3p and -484) is also associated with BMD elsewhere.\(^{24}\) Namely, Gautvik and colleagues\(^{24}\) found a complex relationship between these miRNAs and BMD, which exhibited different effect depending on the skeletal site. Wang and colleagues\(^{50}\) described that miR-484 is involved in mitochondrial fission in cardiomyocytes and adrenocortical cancer cells, which is controlled by FOXO3A and FIS1, demonstrating that miR-484 has a role in controlling bioenergetic homeostasis.
In regard to potential bone-related pathways, miR-223-3p was found to affect HIF-1 signaling pathway, which is described to regulate osteocyte-mediated osteoclastic differentiation by promoting RANKL expression through the activation of JAK2/STAT3 pathway. Indeed, others have reported that miR-223 can modulate the expression of other bone markers such as Ctsk, Runx2, Bglap, Alpl, and Spp1. In addition, this miRNA is involved in the suppression of cell proliferation by targeting

| miRNAs   | KEGG pathways                                      | n<sup>a</sup> | p Value | Genes (n) |
|----------|----------------------------------------------------|---------------|---------|-----------|
| miR-484  | TarBase V.7:                                        |               |         |           |
|          | • Fatty acid biosynthesis                           | 11            | <0.0001 | 2         |
|          | • Fatty acid metabolism                             | <0.0001       | 4       |           |
|          | • Metabolic pathways                                | <0.0001       | 40      |           |
|          | • Steroid biosynthesis                              | <0.0001       | 3       |           |
|          | • Fat digestion and absorption                      | <0.0001       | 4       |           |
|          | • Primary bile acid biosynthesis                    | <0.0001       | 3       |           |
|          | • Vitamin digestion and absorption                  | 0.0001        | 4       |           |
|          | • Fatty acid degradation                            | <0.0001       | 2       |           |
|          | • Retinol metabolism                                | <0.0001       | 6       |           |
|          | • Glycosaminoglycan biosynthesis (heparan sulfate/heparin) | 0.001 | 2       |           |
|          | • Long-term depression                              | 0.037         | 4       |           |
| miR-223-3p | TarBase V.7:                                       |               |         |           |
|          | • Caffeine metabolism                               | 11            | <0.001  | 2         |
|          | • Carbohydrate digestion and absorption             | 0.002         | 4       |           |
|          | • Central carbon metabolism in cancer               | 0.002         | 5       |           |
|          | • Fat digestion and absorption                      | 0.016         | 2       |           |
|          | • Proteoglycans in cancer                           | 0.016         | 8       |           |
|          | • Steroid biosynthesis                              | 0.017         | 2       |           |
|          | • Complement and coagulation cascades               | 0.017         | 7       |           |
|          | • Synthesis and degradation of ketone bodies        | 0.023         | 3       |           |
|          | • Chagas disease (American trypanosomiasis)         | 0.032         | 6       |           |
|          | • HIF-1 signaling pathway                           | 0.032         | 7       |           |
|          | • Hepatitis B                                       | 0.040         | 6       |           |
|          | TargetScan:                                         |               |         |           |
|          | • Inositol phosphate metabolism                     | 0.006         | 2       |           |
|          | • Cytokine-cytokine receptor interaction            | 0.006         | 2       |           |
|          | • Phosphatidylinositol signaling system              | 0.019         | 2       |           |
|          | • Lysine biosynthesis                               | 0.042         | 1       |           |
|          | • Tryptophan metabolism                             | 0.042         | 1       |           |
|          | • Metabolic pathways                                | 0.042         | 6       |           |
|          | • Base excision repair                              | 0.042         | 1       |           |
| miR-328-3p | Tarbase V.7:                                       |               |         |           |
|          | • Biosynthesis of unsaturated fatty acids           | 5             | <0.0001 | 2         |
|          | • Ascorbate and aldarate metabolism                 | 0.005         | 4       |           |
|          | • ECM-receptor interaction                         | 0.013         | 3       |           |
|          | • Fatty acid metabolism                             | 0.013         | 2       |           |
|          | • Primary bile acid biosynthesis                    | 0.021         | 1       |           |
|          | TargetScan:                                         |               |         |           |
|          | • Glycosaminoglycan biosynthesis (heparan sulfate/heparin) | 0.0001 | 3       |           |
|          | • GABAergic synapse                                 | <0.0001       | 3       |           |
|          | • Nicotine addiction                                | <0.0001       | 3       |           |
|          | • Morphine addiction                                | <0.0001       | 3       |           |
|          | • Mucin type O-Glycan biosynthesis                  | 0.002         | 1       |           |
|          | • Transcriptional misregulation in cancer           | 0.003         | 4       |           |

This table demonstrates the predicted affected KEGG pathways (Tarbase V.7 and TargetScan) at p values <0.05 using DIANA – miRPath V.3 with the following settings: pathways union, false discovery rate (FDR) correction box checked, and conservative stats box unchecked.

<sup>a</sup>Numbers of experimentally (Tarbase V.7) and non-experimentally (Target Scan) targeted pathways.
Table 4. Biochemical and Cellular Signaling Pathways Predicted to Be Affected by Morphine-Associated Downregulated miRNAs in Male Mice

| miRNAs          | KEGG pathways                                      | n  | p Value   | Genes (n) |
|-----------------|----------------------------------------------------|----|-----------|-----------|
| miR-200b-3p     | TarBase V.7:                                        |    |           |           |
|                 | - Steroid biosynthesis                             | 1  | 0.014     | 1         |
|                 | TargetScan:                                        |    |           |           |
|                 | - Metabolic pathways                               | 4  | 0.015     | 3         |
|                 | - Pyrimidine metabolism                            | 0.041 | 1         |
|                 | - Purine metabolism                                | 0.046 | 1         |
|                 | - RNA polymerase                                   | 0.046 | 1         |
| miR-28a-3p      | TarBase V.7:                                        |    |           |           |
|                 | - No experimentally validated targets found.       | 0  | NA        | NA        |
|                 | TargetScan:                                        |    |           |           |
|                 | - Cysteine and methionine metabolism               | 5  | <0.0001   | 1         |
|                 | - Selenocompound metabolism                         | 0.0001 | 1         |
|                 | - Glycine, serine, and threonine metabolism        | 0.033 | 1         |
|                 | - ABC transporters                                 | 0.033 | 1         |
|                 | - Biosynthesis of amino acids                      | 0.042 | 1         |
|                 | - Bile secretion                                   | 0.042 | 1         |
| miR-182-5p      | TarBase V.7:                                        |    |           |           |
|                 | - No experimentally validated targets found.       | 0  | NA        | NA        |
|                 | TargetScan:                                        |    |           |           |
|                 | - ECM-receptor interaction                         | 4  | <0.0001   | 3         |
|                 | - Taurine and hypotaurine metabolism               | 0.045 | 1         |
|                 | - Hedgehog signaling pathway                       | 0.045 | 3         |
|                 | - Long-term depression                             | 0.045 | 1         |
| miR-6769b-5p    | TarBase V.7:                                        |    |           |           |
|                 | - No experimentally validated targets found.       | 0  | NA        | NA        |
|                 | TargetScan:                                        |    |           |           |
|                 | - Thyroid hormone synthesis                        | 4  | <0.0001   | 1         |
|                 | - Glycosaminoglycan biosynthesis (keratan sulfate) | 0.004 | 1         |
|                 | - RNA degradation                                  | 0.047 | 2         |
|                 | - Primary immunodeficiency                         | 0.047 | 1         |
| miR-351-5p      | TarBase V.7:                                        |    |           |           |
|                 | - Lysine degradation                               | 1  | 0.0001    | 1         |
|                 | TargetScan:                                        |    |           |           |
|                 | - Glycosphingolipid biosynthesis (lacto and neolacto series) | 2 | <0.0001 | 2         |
|                 | - Mucin type O-Glycan biosynthesis                 | 0.001 | 1         |
| miR-203-3p      | TarBase V.7:                                        |    |           |           |
|                 | - Adherens junction                                | 4  | <0.001    | 4         |
|                 | - Lysine degradation                               | 0.008 | 3         |
|                 | - Degradation of aromatic compounds                | 0.013 | 1         |
|                 | - Protein processing in endoplasmic reticulum     | 0.035 | 4         |
|                 | TargetScan:                                        |    |           |           |
|                 | - Bile secretion                                   | 1  | 0.003     | 1         |
| miR-7002-5p     | TarBase V.7:                                        |    |           |           |
|                 | - No experimentally validated targets found.       | 0  | NA        | NA        |
|                 | TargetScan:                                        |    |           |           |
|                 | - Metabolism of xenobiotics by cytochrome P450     | 6  | <0.0001   | 1         |
|                 | - Tight junction                                   | 0.0001 | 6         |
|                 | - Leukocyte transendothelial migration             | 0.001 | 5         |
|                 | - Cell adhesion molecules (CAMs)                   | 0.002 | 4         |
|                 | - NOD-like receptor signaling pathway              | 0.004 | 1         |
|                 | - Arachidonic acid metabolism                      | 0.007 | 1         |
| miR-200c-3p     | TarBase V.7:                                        |    |           |           |
|                 | - Intestinal immune network for IgA production    | 2  | 0.005     | 1         |
|                 | - Inflammatory bowel disease (IBD)                 | 0.005 | 1         |
|                 | TargetScan:                                        |    |           |           |
|                 | - Metabolic pathways                               | 4  | 0.015     | 3         |
|                 | - Pyrimidine metabolism                            | 0.041 | 1         |

(Continues)
Igf1r, which is related to skeletal response to mechanical loading. These findings, in addition to our finding of reduced expression of Stat3 and Igf1r in bone, suggest that miR-223 may be involved in morphine-induced bone loss in males. Previous evidence also demonstrated a link between miR-223-3p and adipose tissue. Macartney-Coxson and colleagues observed downregulation of miR-223-3p expression in the omentum and subcutaneous adipose tissue after weight loss induced by a gastric bypass. Whether miR-223-3p contributed to morphine-induced metabolic phenotypes in our mice is unknown. We found fatty acid metabolism pathways in morphine-treated male mice was targeted by the upregulated miRNAs. In fact, miRNA data indicated that sustained morphine treatment affected fatty acid metabolism pathways in both sexes, which is consistent with the changes in energy expenditure and respiratory quotient during metabolic assessment. However, our data also suggest that the morphine-induced changes in fat metabolism may occur through distinct mechanisms between sexes.

It is recognized that metabolic disturbances are associated with disruption of bone anabolic pathways (eg, Wnt signaling, parathyroid hormone signaling, insulin, and peroxisome proliferator-activated receptor γ), resulting in impairment of osteoblast function and uncoupling of bone turnover. Skeletal cell fate is regulated by the nutritional environment, and lipids are an essential energy source to bone cells. Osteoblasts also require fatty acid oxidation for normal bone acquisition. Van Gastel and colleagues also demonstrated that lipid scarcity leads skeletal progenitor cell into chondrogenic over osteogenic lineage differentiation. Although chondrocytes are highly

| miRNAs          | KEGG pathways                                      | n^a | p Value | Genes (n) |
|-----------------|----------------------------------------------------|-----|---------|-----------|
| miR-125b-5p     | • Purine metabolism                               | 0.046 | 1       |
|                 | • RNA polymerase                                  | 0.046 | 1       |
|                 | • Lysine degradation                              | 21   | <0.001  | 10        |
|                 | • Caffeine metabolism                             | 0.001 | 2       |
|                 | • MAPK signaling pathway                          | 0.001 | 39      |
|                 | • Phosphatidylinositol signaling system           | 0.001 | 16      |
|                 | • Proteoglycans in cancer                         | 0.001 | 28      |
|                 | • Colorectal cancer                               | 0.002 | 14      |
|                 | • Glycosphingolipid biosynthesis (lacto and neolacto series) | 0.002 | 2   |
|                 | • Endocytosis                                      | 0.002 | 34      |
|                 | • Glycosaminoglycan biosynthesis (heparan sulfate/heparin) | 0.003 | 4   |
|                 | • Adherens junction                               | 0.003 | 18      |
|                 | • N-Glycan biosynthesis                           | 0.004 | 7       |
|                 | • GnRH signaling pathway                          | 0.004 | 18      |
|                 | • Protein processing in endoplasmic reticulum     | 0.004 | 29      |
|                 | • Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.005 | 11    |
|                 | • Glycosphingolipid biosynthesis (ganglio series)  | 0.017 | 3       |
|                 | • Glioma                                           | 0.017 | 11      |
|                 | • Pancreatic cancer                               | 0.022 | 14      |
|                 | • ErbB signaling pathway                          | 0.028 | 15      |
|                 | • Steroid biosynthesis                            | 0.042 | 2       |
|                 | • Vitamin B6 metabolism                           | 0.047 | 2       |
|                 | • Pathways in cancer                              | 0.047 | 18      |
|                 | TarBase V.7:                                      |       |         |           |
|                 | • Glycosphingolipid biosynthesis (lacto and neolacto series) | 3    | 0.0001  | 2       |
|                 | • Mucin type O-Glycan biosynthesis                 | <0.001 | 1      |
|                 | • Glycerophospholipid metabolism                   | 0.005 | 1       |
|                 | TargetScan:                                       |       |         |           |
|                 | • Glycosphingolipid biosynthesis (lacto and neolacto series) | 3    | <0.0001 | 1       |
|                 | • Intestinal immune network for IgA production     | 0.007 | 1       |
|                 | • Inflammatory bowel disease (IBD)                | 0.007 | 1       |
|                 | • Maturity onset diabetes of the young             | 0.019 | 1       |
|                 | TarBase V.7:                                      |       |         |           |
|                 | • Sphingolipid metabolism                         | 6     | <0.0001 | 1       |
|                 | • Glycerolipid metabolism                         | 0.043 | 1       |
|                 | • Ether lipid metabolism                          | 0.043 | 1       |
|                 | • Fc gamma R-mediated phagocytosis                 | 0.043 | 1       |
|                 | • Fat digestion and absorption                    | 0.043 | 1       |
|                 | • Choline metabolism in cancer                    | 0.043 | 1       |
|                 | TargetScan:                                       |       |         |           |
|                 | • Glycosphingolipid biosynthesis (lacto and neolacto series) | 3    | 0.0001  | 1       |
|                 | • Mucin type O-Glycan biosynthesis                 | <0.001 | 1      |
|                 | • Glycerophospholipid metabolism                   | 0.005 | 1       |

NA = not applicable.

This table demonstrates the predicted affected KEGG pathways Tarbase V.7 and TargetScan) at p values <0.05 using DIANA – miRPath V.3 with the following settings: pathways union, false discovery rate (FDR) correction box checked, and conservative stats box unchecked.

^a Numbers of experimentally (Tarbase V.7) and non-experimentally (Target Scan) pathways.
glycolytic, energy production in osteoblasts mainly relies on a higher rate of fatty acid oxidation, not glucose oxidation.\(^{(55)}\) In face of this, we speculate that the changes in circulating miRNA profile and in substrate utilization observed in males after sustained morphine delivery might be contributing to the poor metabolic control of bone mineralization process causing reduction in bone mass\(^{(58)}\); however, additional studies would be necessary to confirm this mechanism.

Among the downregulated miRNAs identified, there is evidence connecting miR-351-5p,\(^{(32)}\) -203-3p,\(^{(33,34)}\) -200c-3p,\(^{(35,36)}\) -125b-5p\(^{(37)}\) to osteogenesis (Table 2). Interestingly, we found that miR-125-5p has affected the greatest number of pathways in males exposed to morphine. There is evidence in the literature that miR-125-5p is associated with bone but in conditions different from our study. miR-125b-5p expression is described to be sex-dependent\(^{(37)}\) and is downregulated in osteoporotic postmenopausal women compared with non-osteoporotic ones.\(^{(38)}\) Moreover, miR-125b-5p\(^{(59)}\) and miR-200b-3p\(^{(60)}\) are miRNAs described to be involved in steroidogenesis. It is reported that miR-125b-5p expression is decreased in polycystic ovary syndrome (PCOS) women,\(^{(39)}\) whereas miR-200b-3p targets steroidogenic pathway enzyme (eg, CYP19A1) that produces estradiol.\(^{(60)}\)

These findings suggest that miR-125b-5p and -200b-3p might unveil potential mechanisms of how morphine may impact steroid production. Here, we have not explored circulating steroid hormone levels and their receptors in our mouse model to understand to what extent the changes in this sex hormone’s levels influenced our bone phenotype, and we believe that such aspect should be considered moving forward. On the other hand, we have identified systemic metabolic alterations associated with morphine treatment that may have affected bone homeostasis.

Finally, future work should examine the nervous system and other potential sources of circulating miRNA changes. Since the nervous system is the major target of opioids, it is possible that the overwhelming suppression miRNAs in males comes from altered secretion by neurons. Although efficacy of opioids for pain is linked to suppression of neural transmission, opioids may also stimulate (directly or indirectly) sympathetic nervous system pathways, which could also indirectly modulate miRNA or catecholamine release to influence bone and metabolism. In summary, we found that sustained morphine delivery leads to reduced osteoblast functionality and lower bone density in male mice, but bone microarchitecture was preserved in female mice after chronic morphine exposure. We also observe that morphine influences substrate utilization and fatty acid metabolism and that these are associated with the upregulation of miR-484, -223-3p, and -328-3p expression in males, which is a potential link between morphine and altered metabolic control of bone mineralization process. Our novel findings have set a precedent for future investigations into how morphine-induced metabolic changes influence bone formation could lead to clinical mitigation strategies for preventing the adverse effects of opioids on bone health.

**Disclosures**

All authors state that they have no conflicts of interest.

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Author Contributions

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All data is available from the corresponding authors upon reasonable request.

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