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The PYY/Y2R-Deficient Male Mouse is Not Protected from Bone Loss Due to Roux-en-Y Gastric Bypass

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Abstract

Background: Peptide YY (PYY) is an anorexigenic gut hormone that also has anti-osteogenic effects, inhibiting osteoblastic activity and inducing catabolic effects. It has been postulated that increases in PYY after Roux-en-Y gastric bypass (RYGB) contribute to declines in bone mineral density (BMD) and increases in bone turnover. The aim of this study is to determine the role of the PYY Y2-receptor in mediating bone loss post-RYGB in mice.

Methods: We compared adult male wildtype (WT) and PYY Y2 receptor-deficient (KO) C57BL/6 mice that received RYGB (WT: n=8; KO: n=9), with sham-operated mice (Sham; WT: n=9; KO: n=10) and mice that were food-restricted to match the weights of the RYGB-treated group (Weight-Matched, WM; WT: n=7; KO: n=5). RYGB or sham surgery was performed at 15–16 weeks of age, and mice sacrificed 21 weeks later. We characterized bone microarchitecture with micro-computed tomography (μCT) at the distal femur (trabecular) and femoral midshaft (cortical). Differences in body weight, bone microarchitecture and biochemical bone markers (parathyroid hormone, PTH; C-telopeptide, CTX; and type 1 procollagen, P1NP) were compared using 2-factor ANOVA with Tukey's adjustments for multiple comparisons.

Results: Body weights were similar in the WT-RYGB, WT-WM, KO-RYGB, and KO-WM: 41–44g; these groups weighed significantly less than the Sham surgery groups: 55–57g. Trabecular BMD was 31–43% lower in RYGB mice than either Sham or WM in WT and KO groups. This deficiency in trabecular bone was accompanied by a lower trabecular number (19%−23%),

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thickness (22%−30%) and increased trabecular spacing (25%–34%) in WT and KO groups ($p<0.001$ for all comparisons vs. RYGB). RYGB led to lower cortical thickness, cortical tissue mineral density, and cortical bone area fraction as compared to Sham and WM in WT and KO groups ($p\leq0.004$ for all). There were no interactions between genotype and bone microarchitecture, with patterns of response to RYGB similar in both WT and KO groups. CTX and P1NP were significantly higher in RYGB mice than WM in WT and KO groups. PTH did not differ among groups.

Conclusions: RYGB induced greater trabecular and cortical deficits and high bone turnover than observed in weight-matched mice, with a similar pattern in the WT and Y2RKO mice. Thus, skeletal effects of RYGB are independent of weight loss, and furthermore, PYY signaling through Y2R is not a key mediator of bone loss post-RYGB.

Keywords

Bariatric surgery; bone loss; bone microarchitecture; bone biomarker; mouse models

1. Introduction

Among U.S. adults, the prevalence of obesity has reached more than a third of the total population[1]. Bariatric surgery has proven to be the most effective treatment of obesity and obesity-related comorbidities such as type 2 diabetes mellitus[2]. In the US alone, there were approximately 252,000 bariatric procedures performed in 2018, a number expected to increase in the setting of up-trending obesity rates and emerging strong evidence of efficacy and safety for these procedures[3]. Although there are overwhelming cardiometabolic benefits, there is also increasing evidence that bariatric surgery leads to a rapid reduction in bone mineral density (BMD), an increase in bone turnover markers as well as increased fracture risk[4].

The two most common procedures used currently are vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB), both of which lead to significant weight loss and metabolic improvements that often last for years if not decades [5]. Both preclinical and clinical studies have shown that calcium and vitamin D supplementation are insufficient in preventing bone loss post bariatric surgery [6, 7]. Rapid and drastic changes in levels of gut hormones such as Glucagon-like peptide-1 (GLP-1) and Peptide YY (PYY) are trademarks of both VSG and RYGB, but their causal role in producing weight loss and metabolic improvements is controversial [8, 9]. Namely, RYGB in GLP-1R and PYY/Y2R- knockout mice is as effective as in wildtype mice in reducing body weight and improving metabolism [10, 11]. Nonetheless, post-surgical alterations in gut hormones may play a role in skeletal effects [7, 12–14]. One of the most significant hormonal changes following RYGB is the elevation in PYY levels. PYY is an anorexigenic gut hormone secreted from intestinal L-cells in response to nutrient stimulation to promote satiety. Postprandial serum PYY is proportional to caloric intake thus increasing satiety.

Several lines of evidence suggest that PYY may have a direct negative impact on the skeleton. In human studies, a cross-sectional evaluation of healthy premenopausal women showed a negative association between hip BMD and PYY levels [15]. Clinical studies of

women with anorexia nervosa have shown an inverse association between elevated PYY levels and lower spinal BMD and reduced bone formation serum marker of pro-collagen type I N-terminal propeptide (P1NP) [16]. Conversely, obesity has been associated with lower PYY levels and greater BMD suggesting an inverse relationship between serum PYY levels and bone homeostasis especially under conditions that alter energy balance [15–18]. Following RYGB, fasting and postprandial PYY levels are considerably elevated compared to peak levels in lean and obese individuals [19, 20]. Bone loss is also significant post bariatric surgery [21, 22], while interestingly following gastric banding which does not alter PYY levels [23, 24], bone loss is less pronounced [25]. Indeed, PYY has been correlated with changes in bone resorption markers and bone density after gastric bypass[7, 13]. However, causal evidence linking post-surgical PYY elevations to changes in bone mass are currently lacking.

Murine models also suggest that the importance of PYY signaling to bone health. Male and female PYY knockout (KO) mice exhibit enhanced osteoblast activity and greater trabecular bone mass, and conversely mice with PYY overexpression display diminished osteoblast activity in [26]. Another study demonstrated adult PYY KO mice had increased bone mineral content with increased mineralization of both cortical and trabecular compartments [12]. Moreover, systemic PYY acts through various Y receptors. PYY signaling through Y2 receptors in the brain and periphery is thought to reduce hunger and food intake in rodents [27] and its role in bone homeostasis was revealed with the demonstration of increased bone formation in the distal femur (trabecular) of mice in germ line deletion of Y2 receptors [28]. Furthermore, deletion of hypothalamic Y2 receptors in adult mice has been associated with marked elevation in cortical bone formation [29]. In a study by Seldeen et al, ovariectomized mice treated with a brain penetrant Y2 receptor small molecule antagonist showed reduced weight, increased whole-body BMD and a decrease in bone resorption biomarkers, C-terminal telopeptide 1 (CTX-1) [30]. Although this evidence suggests a negative association between PYY signaling via Y2-receptor and bone homeostasis, to the best of our knowledge, no study has assessed the link between PYY Y2-receptor in mediating bone loss post RYGB.

The aim of this study is to determine the role of the PYY Y2-receptor in mediating bone loss post-RYGB using a murine model.

2. Materials and methods

a. Animals

We evaluated wild-type and Y2 receptor (Y2R) deficient mice that were part of a larger study examining the role of Y2R signaling on body weight and body composition, food intake and food choice, energy expenditure, glucose tolerance and insulin sensitivity post RYGB surgery[10]. Constitutive Y2R-knockout mice (C57BL/6NTac-*Npy2r*^{em3978Tac}; Y2RKO) were generated at Taconic (San Diego, CA, USA). Wild-type mice (C57BL/6) were obtained from heterozygous breeding pairs housed at Jackson Labs (Bar Harbor, ME, USA). Mice were housed in standard caging at 22 °C in a 12-h light:12-h dark cycle at standard temperature and humidity conditions with *ad libitum* access to water and food (except where noted). Starting at about 6 weeks of age, mice were exposed to a two-choice

diet consisting of a 60% high-fat (HF) diet (Kcal%: Carb, 20; Fat, 60; Prot, 20, Diet D12492, Research Diets, New Brunswick, NJ, USA) and 10% low-fat (LF) diet (Kcal%: Carb, 58; Fat, 13; Prot, 28.5, 5001, Purina LabDiet, Richmond, IN, USA) for the duration of the experiment. The rationale for the two-choice diet was to better mimic the human situation, to measure food choice, and that mice eat relatively more chow immediately after RYGB to maintain motility of the small gastric pouch. All animal studies were conducted according to protocols reviewed and approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee (IACUC).

b. Surgery and specimen preparation

The cohort of mice consisted of 24 adult male wildtype (WT) and 24 adult male Y2 receptor knock out (KO) mice. Mice of each genotype at approximately 16 weeks of age were stratified into 3 groups: Roux-en-Y gastric bypass surgery (RYGB) [WT: n=8, KO: n=9], sham surgery (Sham) [WT: n=9, KO: n=10] and weight-matched to RYGB by calorie restriction (WM) [WT: n=7, KO: n=5]. At 15 to 16 weeks of age, the RYGB mice underwent a jejuno-gastric anastomosis: the cut end of the mid jejunum was connected to a small gastric pouch and the other end of the cut jejunum was anastomosed to the lower jejunum, resulting in a 5–6 cm long Roux limb, a 9–11 cm long biliopancreatic limb, and a 20–25 cm long common limb. Sham surgery consisted of laparotomy only, without transection of jejunum. Mice weight-matched to the RYGB group were initially restricted to about 50–70% of the calorie intake of the RYGB group. Pre-weighed amounts of food (Kcal:~93% high-fat, ~7% chow) were given once per day during the light period. At approximately 35 to 37 weeks of age (21 weeks after surgery), mice were food deprived for 3–5 h and euthanized by decapitation, after which the femur was harvested, and trunk blood was collected. Plasma parathyroid hormone (PTH) was assessed by ELISA (Immutopics, San Clemente, CA), and plasma levels of the bone resorption marker, type 1 collagen C-telopeptide (CTX) and bone formation marker, amino-terminal propeptide of type I procollagen (P1NP) were measured using mouse ELISA kits (IDS, Fountain Hills, AZ).

a. μCT analysis of bone microarchitecture

Micro-computed tomographic (μCT) imaging was performed of the femoral distal metaphysis and mid-diaphysis using a benchtop imaging system (μCT40, Scanco Medical, AG, Brüttisellen, Switzerland). Scans were acquired using 10 μm^3 isotropic voxel size, 70 kVp peak potential, 114 μA intensity, 200 msec integration time and scanning and analysis was performed in accordance with guidelines for assessment of bone microstructure in rodents [31]. Trabecular bone microarchitecture was evaluated in the endocortical region of the distal femoral metaphysis in a region of interest that started 200μm above the peak of the distal growth plate and extended proximally 1.5mm (150 slices). Cortical bone microarchitecture was also evaluated in the femoral diaphysis in a region that began 55% of the femoral length below the top of the femoral head and extended distally 500μm. Segmentation thresholds of 375 and 700 mg HA/ cm^3 were used for the evaluations of trabecular and cortical bone, respectively. All analyses were carried out using the scanner manufacturer (Scanco Medical) evaluation software. Trabecular bone outcomes included trabecular bone volume fraction (Tb. BV/TV, %), trabecular bone mineral density (Tb. BMD, mg HA/cm^3), trabecular thickness (Tb. Th, mm), trabecular number (Tb. N, mm^{-1}),

trabecular separation (Tb. Sp, mm), connectivity density (Conn.D, mm^{-3}), and structural model index (SMI). Cortical bone outcomes included cortical tissue mineral density (Ct. TMD, mg HA/ cm^3), cortical thickness (Ct. Th, mm), cortical bone area (Ct.Ar, mm^2), total bone area (Tt.Ar, mm^2), cortical bone area fraction (Ct.Ar/ Tt.Ar, %), cortical porosity (Ct.Po, %), polar moment of inertia (pMOI, mm^4), and the maximum and minimum moments of inertia (Imax and Imin, mm^4).

b. Statistical analysis

Differences in body weight, bone microarchitecture and biochemical labs were analyzed with two-way analysis of variance (ANOVA). Within each genotype, we compared means between the 3 treatment groups (RYGB, Sham, Weight-Matched) using Tukey's adjustments for multiple comparisons. *Šidák* adjustments were used to compare sets of means between the two genotypes. All data analyses were performed using GraphPad Prism software version 9.4.0 (San Diego, CA, USA). The threshold of statistical significance was set at $P \leq 0.05$ and data reported as mean ±SD.

3. Results

a. Body weight

Presurgical body weights were similar in the wildtype and Y2 receptor knockout (Y2RKO) mice, assigned to the RYGB, sham surgery (Sham) and weight-matched (WM) groups (Table 1). After surgery, wildtype (WT), RYGB and WM groups had similar body weights (41.0 ± 6.1 g; 42.2 ± 0.5 g, Figure 1) which were significantly lower than the WT- Sham group (57.0 ± 3.4 g; p values <0.0001, Figure 1). A similar pattern was seen among Y2RKO genotype (RYGB 43.8 ± 4.5 g; WM 43.5 ± 0.5 g; Sham 55.0 ± 5.3 g; comparisons vs. Sham p-values <0.0001, Figure 1). There were no differences between wildtype and Y2RKO genotypes in post-surgical body weights.

b. Bone microarchitecture

Within wildtype mice, RYGB group had 41% and 31% lower trabecular BMD (Tb. BMD) in comparison to Sham and WM groups, respectively (Figure 2a; $p \leq 0.01$). There was a similar pattern within Y2RKO with RYGB having a 43% and 39% lower Tb. BMD in comparison to Sham and WM (Figure 1a; $p \leq 0.0001$). The deficiency in trabecular bone among the RYGB mice was accompanied by a lower trabecular number (19%−23%) (Figure 2b), thickness (22%−30%) (Figure 2c) and increased trabecular separation (25%–34%) (Figure 2d) within both the wildtype and Y2RKO mice ($p \leq 0.001$ for all). There was no difference in most trabecular parameters between Sham and WM, except for trabecular thickness which was lower in the WM group as compared to Sham in both genotypes (Figure 2c, $p \leq 0.01$). There were no differences between wildtype and Y2RKO genotypes for RYGB, Sham or WM groups in any of the trabecular parameters (Figure 2a–d).

RYGB led to lower cortical tissue mineral density, cortical thickness, and cortical bone area fraction as compared to Sham and WM in both wildtype and Y2RKO genotypes (Figure 3a–c, $p \leq 0.01$ for all). Wildtype RYGB had nearly 3-fold higher cortical porosity in comparison to Sham or WM (Figure 3d, $p \leq 0.0001$). Cortical porosity was less prominently

observed after RYGB in the Y2RKO mice (WT vs Y2RKO; Figure 3d, p≤0.01) and was not significantly higher than Sham or WM within the Y2RKO group (Figure 3d). There were no differences seen for any of the other cortical parameters between WT and Y2RKO genotypes.

a. Bone turnover markers and PTH

CTX and P1NP were significantly higher in RYGB mice than WM within both Wildtype and Y2RKO mice (Figure 4a,b; p≤0.05). Additionally, there were higher CTX levels within Wildtype RYGB as compared to Sham (Figure 4a; p≤0.05). P1NP was elevated in RYGB group as compared to Sham within both genotypes (Figure 4b; p≤0.0001). The only difference appreciated between Wildtype and Y2RKO groups was seen in P1NP with overall higher levels seen in the Y2RKO mice (Figure 4b; p≤0.0001). PTH did not differ within or between genotypes (Figure 4c).

4. Discussion

This study, the first to examine the role of PYY via Y2 receptor in bone homeostasis post RYGB, shows that loss of signaling via Y2 receptors is not protective in mediating bone loss in mice. Despite experiencing similar post-surgical weight loss, RYGB and WM groups had a dramatically different bone phenotype. Specifically, RYGB mice within each genotype had a significantly lower trabecular vBMD, number, and thickness with greater trabecular spacing, as well as lower cortical TMD, thickness and area along with greater cortical porosity. Furthermore, CTX and P1NP were significantly elevated in RYGB compared to Sham and WM groups, with a similar pattern in both genotypes. The distinctive skeletal pattern observed in the RYGB group implies weight-independent and surgery-specific skeletal effects that are not impacted by Y2 receptor deletion.

These results suggest that PYY signaling through the Y2 receptor does not play a significant role in mediating bone loss after RYGB. Prior clinical studies have shown that elevated PYY is associated with lower bone mass and higher bone turnover in many pathologic conditions, including after bariatric surgery[7, 12, 13, 26, 32]. These studies, however, have been limited by small sample sizes and observational study designs, and are unable to demonstrate causality. Understanding the effects of PYY on bone loss have remained limited, partly due to the presence of multiple Y receptors and their variable downstream effects. PYY binds with differing affinities to receptors of the neuropeptide Y-receptor family (Y1,Y2, Y3, Y4 and Y5 in humans and Y6 in rodents) expressed across a number of different brain regions and central nervous system. While Y1, 4 and 5 receptors are generally considered to be post-synaptic, Y2 is primarily located pre-synaptically and functions as an autoreceptor to inhibit PYY release along with other neurotransmitters thus suggesting a central role for compound action in bone homeostasis [30, 33]. Despite its potential role as a regulator of bone, the Y2 receptor is not located within bone tissue; indeed, prior studies have demonstrated Y1 receptor to be the only Y receptor known to be expressed on osteoblasts [34–36]. Given increasing body of experimental evidence to suggest that bone remodeling is under central, hypothalamic regulation [37], Y2R mediated action is thought to affect bone physiology by decreasing number of progenitors and potentially altering Y1 receptor

expression within bone cells, leading to lower bone density [29]. Here we provide evidence that Y2R mediated action of PYY does not contribute to in bone deficits as evidenced by our finding that Y2RKO mice are not protected against bone loss post-RYGB. It remains unknown, however, whether PYY might still exert RYGB-associated skeletal decline via the bone-specific Y1, or other receptors even in the absence of Y2R's paracrine and central effects.

In our present study, the only significant differences between wildtype and Y2RO genotypes were observed in P1NP levels and cortical porosity within the RYGB groups. P1NP was somewhat higher in Y2RKO-RYGB as compared to wildtype-RYGB, perhaps an indicator that Y2RKO has a global effect of increasing bone turnover. This finding would be consistent with preclinical studies demonstrating increased bone mass in PYY KO mice as well as clinical studies that have shown decreased P1NP levels after post-prandial elevations in PYY [38–40]. Similarly, the increase in cortical porosity after RYGB was less pronounced in the Y2RKO genotype as compared to Wildtype. This finding contrasts with the overall increased cortical porosity observed in PYY KO mice in another study [12]. Our result could imply that Y2RKO provides partial protection from RYGB-induced cortical porosity. Regardless, considering that the bone-sparing phenotype is not seen in any other cortical or trabecular parameters for the Y2RKO-RYGB mice, this partial decrease in cortical porosity elevation is unlikely to have a large overall impact on skeletal strength.

Other mechanisms have been postulated to account for the pathophysiology of post-bariatric decreased bone density and increased fracture risk. Prevailing theories suggest a multitude of factors including nutritional factors, secondary hyperparathyroidism, mechanical unloading, changes in bone marrow adiposity, body composition and gut-hormones to explain RYGB-associated skeletal changes. Although our primary focus was to investigate the actions of the gut-hormone PYY via Y2 receptor, data from our study also allows for examination of the role of weight loss on the post-RYGB skeleton. In our study, despite achieving equivalent body weight in RYGB and WM groups within both genotypes, RYGB had greater bone loss as evidenced by deficits in micro-computed tomography (μCT) parameters as well as elevated bone turnover markers. These data are consistent with the skeletal findings of other murine models of bariatric surgery that have involved weight-matched groups, and suggests that mechanical unloading is not the primary driver in bone loss post-RYGB [41–43] .This phenotype is also consistent with prior longitudinal clinical studies that found that substantial bone loss and deterioration in bone strength continues after weight stabilization [44, 45]. In addition, serum PTH showed did not differ between RYGB, Sham and WM groups nor between genotypes, demonstrating that secondary hyperparathyroidism due to calcium and/or vitamin D malabsorption did not play a significant role in mediating differential bone loss. Similar PTH levels as well as coupling of elevated bone resorption index, CTX and bone formation index, P1NP in the setting of high bone turnover state in RYGB groups as compared to Sham are consistent with prior murine RYGB studies [43].

Our study has several limitations. We used a germline knockout model, thus it is possible that the early absence Y2R-signaling induced compensatory changes that could have obscured normal physiology. Inducible knockout models should therefore be used in future experiments. We did not measure calcium and vitamin D levels, although it was reassuring

that PTH values were similar across all groups within and between genotypes. Although our study evaluated trabecular and cortical bone microarchitecture within the femoral metaphysis and diaphysis, respectively, it is possible that the femur may not reflect the skeletal phenotype at all sites. Thus, future studies might consider performing biomechanical testing and/or examining the microarchitecture of other skeletal sites (e.g. vertebrae) for a more comprehensive understanding of post-RYGB skeletal phenotypes. While our study provided evidence that the Y2R is not involved in RYGB-associated bone loss, PYY activity via the Y1 receptor was not evaluated. It will be important to gather further understanding of PYY action via Y1 receptor on the regulation of bone mass and strength post RYGB, especially given its known direct effects on osteoblast differentiation [34–36] . Future studies might also consider other post-bariatric hormonal changes to determine whether they contribute to deficits in bone microarchitecture post-RYGB. For example, in mouse models of sleeve gastrectomy, circulating GranulocyteColony Stimulating Factor (G-CSF) and intestinal Fibroblast-Growth Factor 15 (FGF15) have been found to be important mediators of skeletal changes [14, 46]; it remains unclear whether these pathways also contribute to gastric bypass associated bone loss. Overall, a more comprehensive understanding of the associations between skeletal homeostasis and gut hormones mediating bone active pathways may be illuminating.

In summary, this study provides important information about bone microarchitecture outcomes and the role of PYY via Y2 receptor post-RYGB, with RYGB mice in both Wildtype and Y2RKO genotypes demonstrating decreased trabecular and cortical bone density and disrupted bone microarchitectural characteristics as compared to weight-matched mice. Our findings suggest that skeletal effects of RYGB are independent of PYY's actions through the Y2 receptor, as well as being independent of weight loss and PTH. Further studies are required to elucidate a more comprehensive understanding of the mechanisms of perturbed bone homeostasis after RYGB.

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Highlights

- Roux-en-Y Gastric Bypass (RYGB) leads to a reduction of cortical and trabecular bone mass, and an increase in markers of bone formation (P1NP) and resorption (CTX) in our murine model
- Peptide YY (PYY) signaling through Y2R is not a key mediator of bone loss post RYGB, as evidenced by similar skeletal phenotypes in the Y2R knockout and wildtype mice
- Skeletal effects of RYGB are independent of weight loss

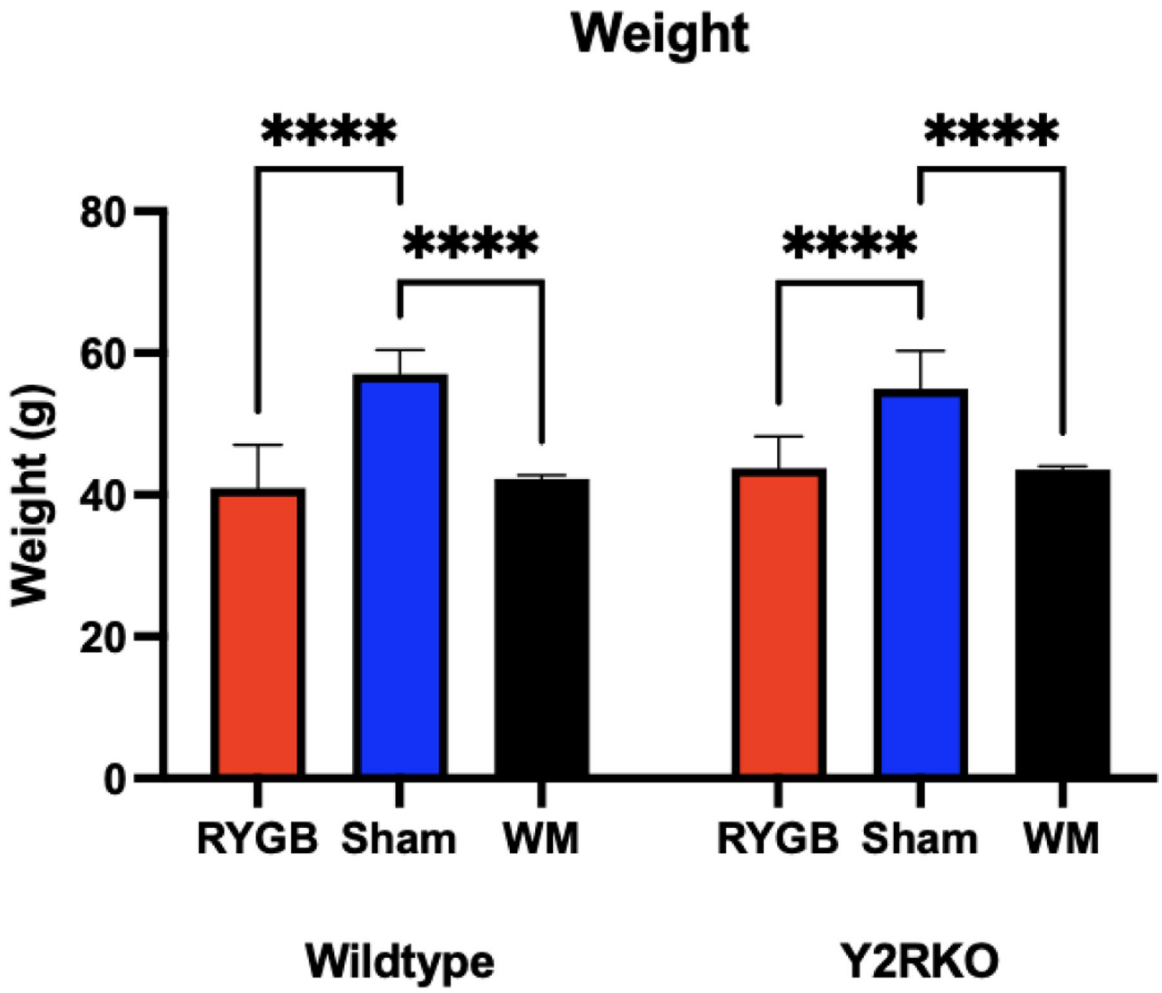

Figure 1-

Post-surgical body weight (g)

Y2RKO: Y2 receptor knockout; RYGB: Roux-en-Y gastric bypass; WM: weight-matched [* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001]

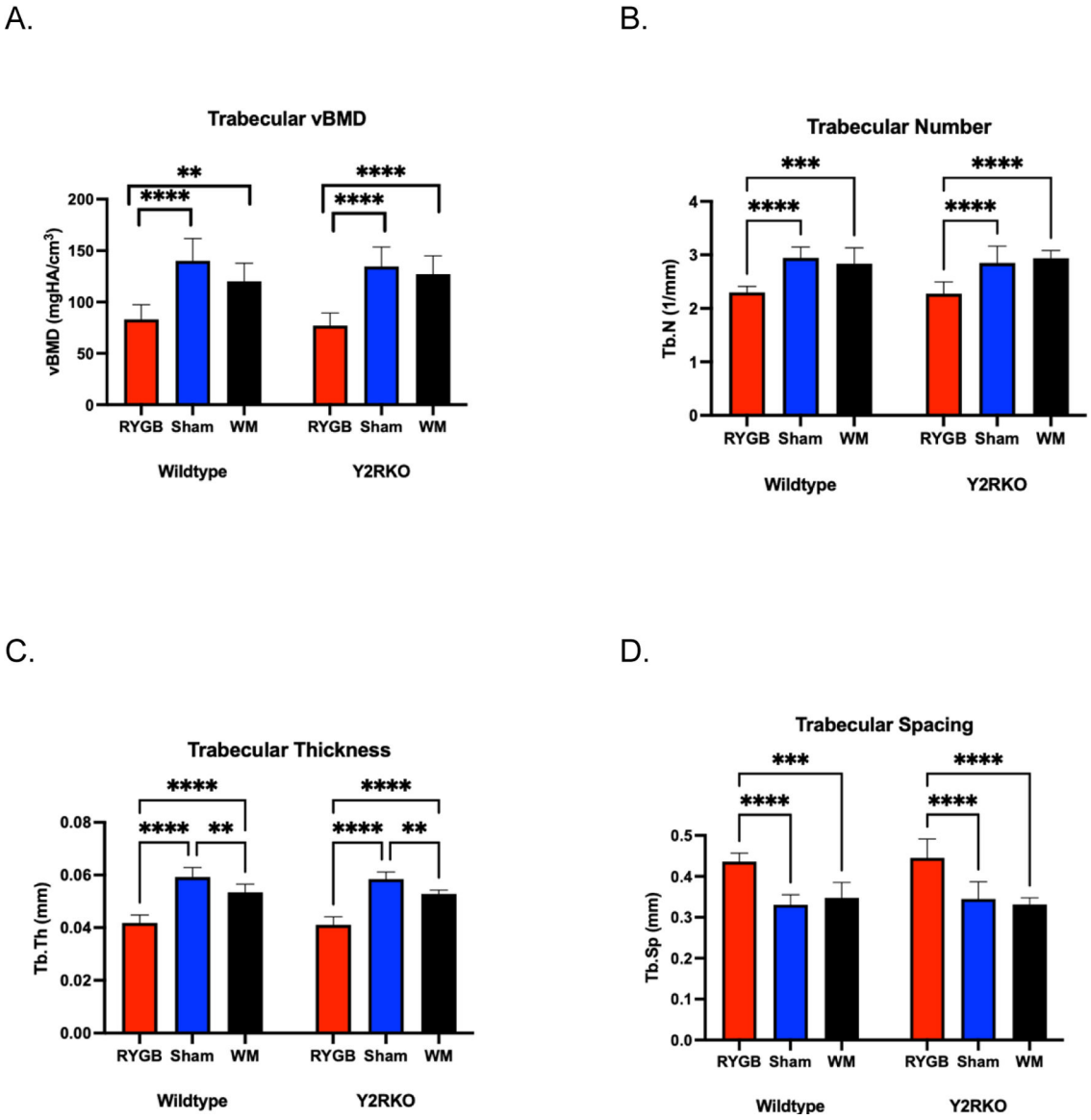

Figure 2.

Effect of RYGB, Sham or WM on micro-computed tomographic (μCT) trabecular parameters. (a) Trabecular vBMD, (b) Trabecular number, (c) Trabecular thickness, (d) Trabecular spacing. [* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001]

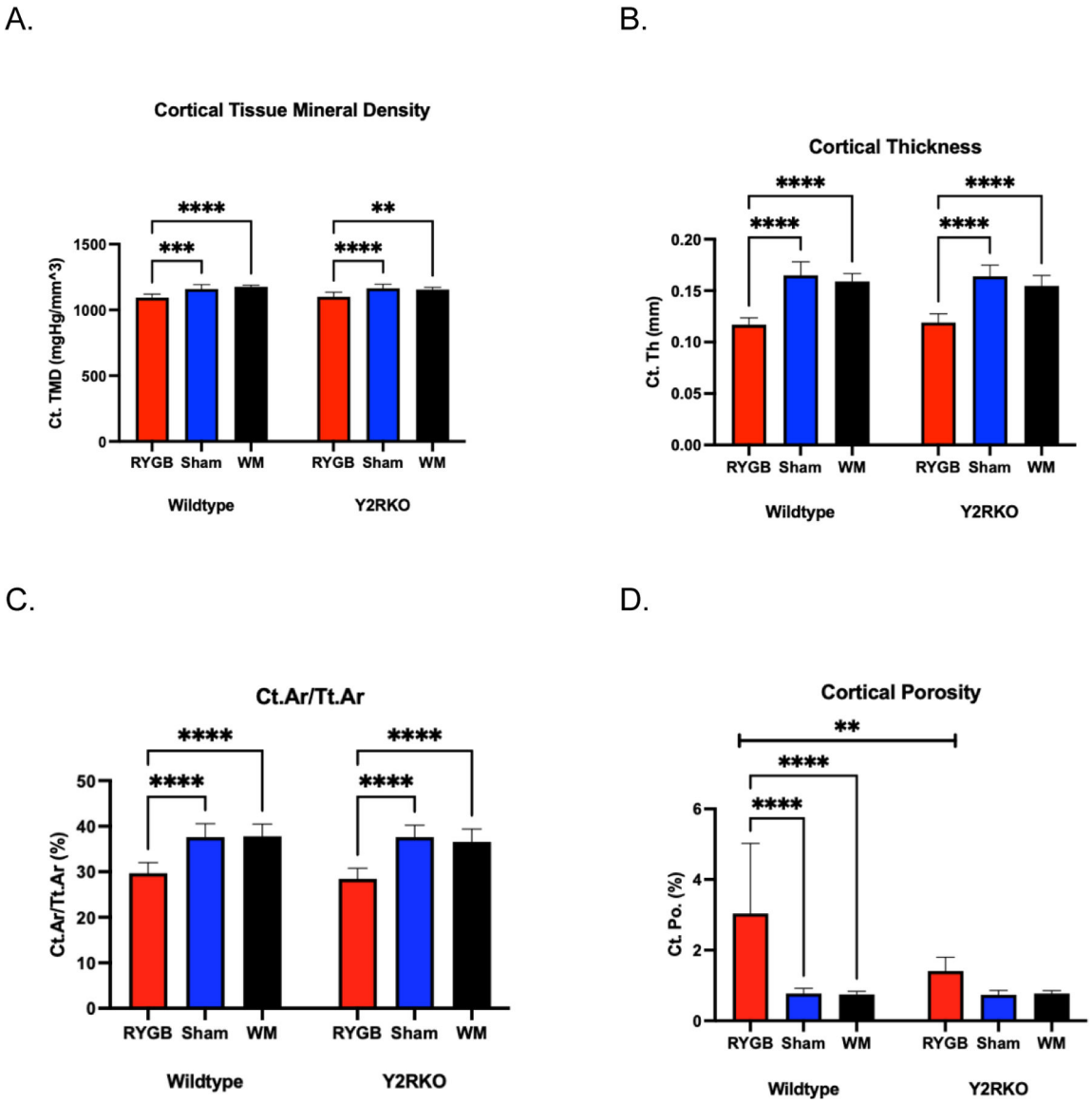

Figure 3.

Effect of RYGB, Sham or WM on micro-computed tomographic (μCT) cortical parameters. (a) Cortical tissue mineral density, (b) Cortical thickness, (c) Cortical bone area fraction, (d) Cortical porosity. [* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001]

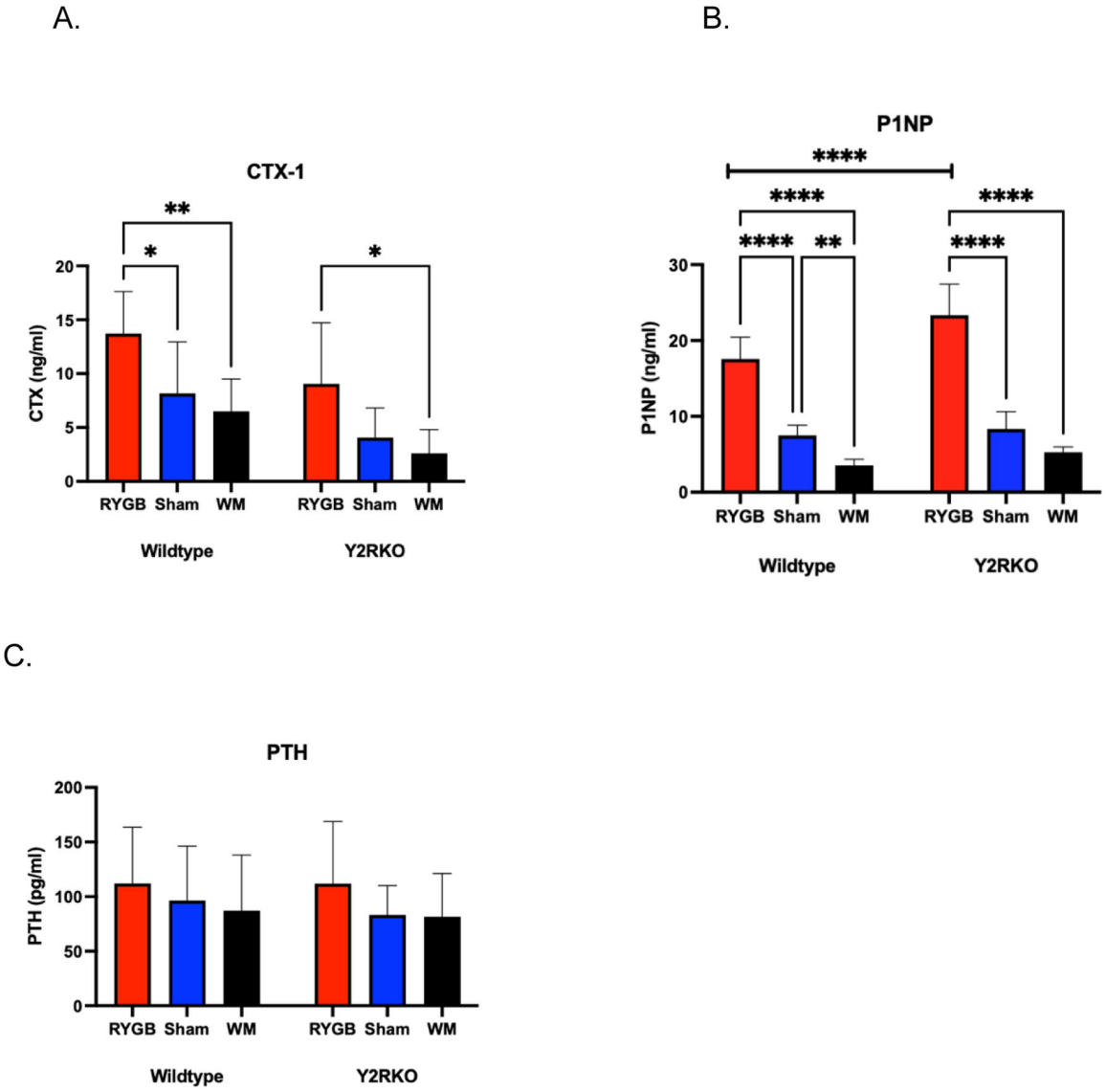

Figure 4.

Effect of RYGB, Sham or WM on bone turnover markers in WT and Y2RKO mice. (a) CTX-1, (b) P1NP, (c) PTH. [* p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001]

Table 1-

Pre-surgical body weight (g)

	Wildtype	Y2RKO
RYGB	45.1 ±3.9	46.0 ±2.0
Sham	45.9 ±2.5	45.9 ± **3.7**
WM	43.3 ±3.0	47.6 ±3.1

Y2RKO: Y2 receptor knockout; RYGB: Roux-en-Y gastric bypass; WM: weight-matched [p>0.05 for all; no statistical significance appreciated within RYGB, sham surgery and weight-matched groups as well as between two genotypes, wildtype and Y2RKO]