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High dose vitamin D supplementation does not rescue bone loss following Roux-en-Y gastric bypass in female rats

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ABSTRACT

Postoperative bone loss and increased fracture risk associated with Roux-en-Y gastric bypass (RYGB) have been attributed to vitamin D/calcium malabsorption and resultant secondary hyperparathyroidism (HPT). Adequate vitamin D supplementation (VDS), particularly in an older female population, reduces incidence of secondary HPT but the effect on bone loss and fracture risk remains unclear. To investigate whether VDS corrects the RYGB bone phenotype, 41 obese adult female rats were randomized to RYGB with 1000 IU (R1000) or 5000 IU (R5000) vitamin D/kg food or a sham surgical procedure with either paired (PF) or ad libitum (AL) feeding. Bone turnover markers, urinary calcium/creatinine ratio (CCR), and serum calciotropic and gut hormones were assessed throughout a 14-week postoperative period. Femurs were analyzed by micro-computed tomography (μCT), three-point bending test, and histomorphometry. 1000 IU animals had low 25-hydroxyvitamin D (25(OH)D), high serum parathyroid hormone (PTH), and very low urine CCR levels. 5000 IU corrected the 25(OH)D and secondary HPT but did not increase urine CCR or serum levels of 1,25-dihydroxyvitamin D (1,25(OH)D) significantly between RYGB groups. Compared to sham animals at 14 weeks, RYGB animals had significantly higher serum osteocalcin (OCN) and C-terminal telopeptide (CTX) levels. The gut hormone peptide tyrosine tyrosine hormone (PYY) was higher in the RYGB groups, and leptin was lower. μCT and biomechanical testing revealed RYGB females had decreased cortical and trabecular bone volume and weaker, stiffer bone than controls. Histomorphometry showed decreased bone volume and increased osteoid volume with increased mineral apposition rate in RYGB compared to controls. No differences in bone phenotype were identified between 1000 IU and 5000 IU groups, and osteoclast numbers were comparable across all four groups. Thus, in our model, 5000 IU VDS corrected vitamin D deficiency and secondary HPT but did not rescue RYGB mineralization rate nor the osteomalacia phenotype. Longer studies in this model are required to evaluate durability of these detrimental effects. Our findings not only underscore the importance of lifelong repletion of both calcium and vitamin D but also suggest that additional factors affect skeletal health in this population.

1. Introduction

Roux-en-Y gastric bypass (RYGB) is the most durable and effective treatment for morbid obesity and its associated complications [1,2]. From 1998 to 2008, almost 750,000 RYGB procedures were performed in the United States, accounting for over 75% of all bariatric operations during this period and reaching over 1 million by 2015 [3]. Of these, it is estimated that over 80% were performed in women and more than two-thirds of these women were over 40 years of age [4,5]. Not surprisingly, skeletal disease and mineral homeostasis have been identified as major post-operative concerns, particularly in this older female demographic [6–8].

Of all bariatric procedures, RYGB has been most clearly shown to have negative skeletal effects [9–11]. Numerous studies have documented impressive increases in bone turnover, with 2–3 fold increases in biochemical markers of bone resorption and more modest increases

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in markers of bone formation [9]. By one year post-RYGB, bone mineral density (BMD) measured by dual-energy X-ray absorptiometry (DXA) dramatically declines at femur (5–11%) and spine (3–7%) sites [12–15], and by two years RYGB patients have lower bone strength and thinner, more porous cortical bone microarchitecture, as evidenced by high-resolution peripheral quantitative computed tomography (HR-pQCT) [16]. These bone fragility concerns have been further corroborated by long-term, population-based studies showing higher fracture risk following bariatric surgery – a risk driven predominantly by RYGB [17–21].

Potential mechanisms that underlie RYGB-associated bone loss include nutritional, hormonal, and mechanical unloading of bone after weight loss. One key driver of bone loss in this population is considered to be calcium and vitamin D deficiency resulting from compromised gut absorption, which can lead to elevations in serum parathyroid hormone (PTH). Serum PTH then acts directly on the bone and kidney and indirectly on the gut to raise and maintain serum calcium concentration. Clinical efforts to improve post-RYGB calcium homeostasis have focused on calcium and vitamin D supplementation, but little research has been done to understand the mechanisms behind the skeletal effects and whether vitamin D supplementation actually mitigates the bone loss. A number of groups have established animal models of RYGB to explore skeletal effects, but these studies have been done in male rodents and without long-term vitamin D supplementation [22–24]. Therefore, we employed a female model of RYGB and two levels of vitamin D supplementation to better characterize the skeletal phenotype, to evaluate if vitamin D supplementation is able to rescue this phenotype, and to explore potential vitamin D-independent mechanisms of bone loss after RYGB.

2. Materials and methods

2.1. Animals, diets, and surgical protocols

Protocols were approved by the University of Florida Institutional Animal Care and Use Committee in accordance with guidelines established by the National Institutes of Health. Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in individual cages at a constant temperature of 21–23 °C with a 12-hour light-dark cycle. To produce diet-induced obesity (DIO), 3-week-old female pups were given ad libitum access for 18 weeks to a 5.2 kcal/g, 60% fat diet (D12492, Research Diets, New Brunswick, NJ) containing 1000 IU of vitamin D3/kg chow. Once DIO was established, rats were randomly assigned to RYGB ($n = 20$) or sham procedure ($n = 21$; Fig. 1A). RYGB was performed as previously described [22,25]. Briefly, a 4-mm enterotomy was made 35 cm proximal to the ileocecal valve (common channel). The bowel was transected 10 cm proximal to this point (Roux limb). An interrupted end-to-side hand-sewn anastomosis of the biliopancreatic limb (25–35 cm length) to the enterotomy was performed using polydioxanone suture. After ligating the left gastric artery and sparing vagal nerves, a small stomach pouch was created, and the gastrojejunostomy of the Roux limb was hand sewn. The defunctionalized stomach was closed in two layers. Sham animals received similar incisions, stomach mobilization, operative time, and closure as RYGB.

2.2. Diet protocols and weight distribution

After the procedure, rats were allowed 2 weeks for return of bowel function. Sham animals were then randomized to an ad libitum group taking a 40% fat diet containing 2% calcium and 1000 IU vitamin D3/kg chow (D11021101, Research Diets) or a pair fed group, in which the same chow was provided in restricted amounts based on the intake of rats in the RYGB group. The RYGB rats were ad libitum fed after randomization to one of two dietary subgroups: the identical diet used in sham animals, either containing 2% calcium and 1000 IU vitamin D3/kg of chow (D11021101, Research Diets), or one containing 2% calcium and 5000 IU vitamin D3/kg and otherwise identical (D14120501, Research Diets). Weekly weights and daily chow and water intake were recorded.

2.3. Specimen collections

Twenty four-hour urine collections were obtained using metabolic cages pre-operatively and every 4 weeks after recovery to measure calcium and creatinine. Pre-operative and monthly dorsal tail bleeds were collected after a 2-hour fast. At the time of euthanasia (using inhaled isoflurane), 2-hour fasting intra-cardiac blood was collected. Blood was analyzed for phosphate, calcium and albumin on an automated system (Dimension Xpand Clinical Chemistry Analyzer, Siemens Healthcare Diagnostics, Inc., Indianapolis, IN). Serum was stored at −20 °C until further use. Femurs were dissected free of excess tissue and placed in cooled 40% ethanol. Femurs were stored at 4 °C until further use.

2.4. Biochemical assays

Rat intact PTH (Immutopics, Inc.; San Clemente, CA), serum 25(OH)D, 1,25(OH)D, OCN, N-terminal propeptide of type I procollagen (P1NP), and CTX immunoassays (Immunodiagnostic Systems, Ltd.; Scottsdale, AZ) were performed using commercially available kits according to manufacturer instructions. Serum insulin, leptin and PYY were measured in duplicate using the Milliplex Rat Gut Hormone Panel (EMD Millipore, Billerica, MA) on the Luminex xMAP platform.

2.5. Micro-computed tomography analysis

Femurs in ethanol were scanned using a Scanco microCT-35 (Scanco Medical AG, Brutisellen, Switzerland) at 50 keV and 200 mA using a 0.5 mm aluminum filter with an isotropic voxel size of 10 μm connected to an HP Integrity 64-bit server running the Open Virtual Memory System, as previously described [26].

2.6. Three-point bending test

All femurs were loaded to failure in three-point bending. All whole bone tests were conducted by loading the femur in the posterior to anterior direction, such that the anterior quadrant was subjected to tensile loads. The lower supports of the three-point bending apparatus were 20 mm wide. Tests were conducted with a deflection rate of 0.08 mm/s using a servohydraulic testing machine (Instron model 8874; Instron Corp., Norwood, MA, USA). The load and mid-span deflection were acquired directly at a sampling frequency of 200 Hz. Load-deflection curves were analyzed for stiffness, maximum load, and work to fracture. Yield is defined as a 10% reduction in the secant stiffness (load range normalized for deflection range) relative to the initial tangent stiffness. Post-yield deflection, defined as the deflection at failure minus the deflection at yield, was measured also. Femurs were tested at room temperature and kept moist with phosphate-buffered saline.

2.7. Histomorphometry

Calcein (Sigma-Aldrich #C0875; 30 mg/kg body weight) was administered by intra-peritoneal injection 8 and 2 days prior to euthanasia. Femurs were stripped of soft tissue and fixed in 70% ethanol, dehydrated and cleared of non-mineralized tissue, then infiltrated at 4 °C with methylmethacrylate/dibutyl phthalate (85%/15%) and increasing concentrations of benzoyl peroxide (0–2.5%) for 8 days. Following infiltration, bones were embedded in MMA/DP with 2.5% BP and polymerized in radiant heat (37 °C). Femurs were cut using a Leica 2265 microtome fitted with a 160 mm D-profile tungsten-carbide knife

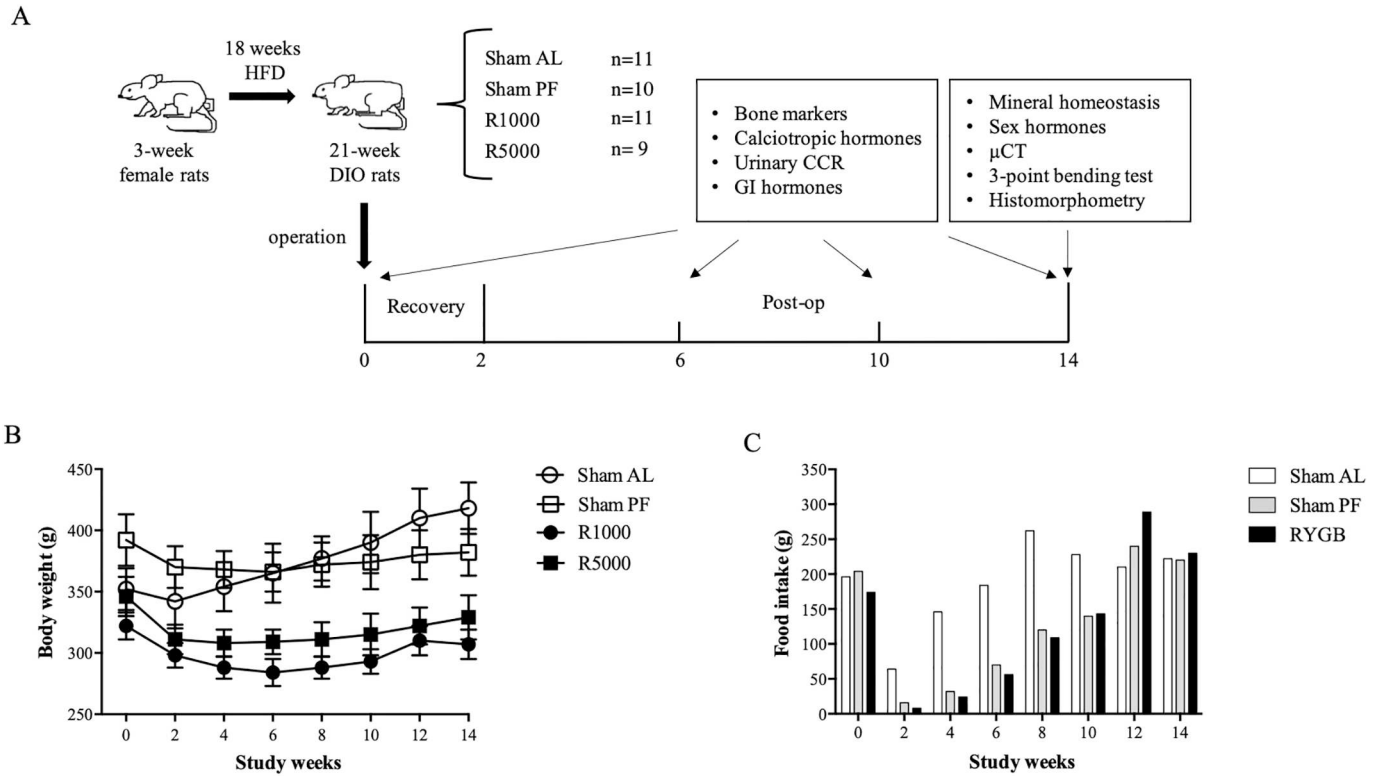

Fig. 1. Study overview, weight, and food intake in DIO female rats after sham operation or RYGB. (*A*) Diagram of study overview. (*B*) Changes in body weight over study duration in sham PF, R1000 and R5000 groups. (*C*) Weekly food intake over study duration. Food intake was similar for both RYGB groups, so data pooled for presentation. Data presented as means ± SEM.

(Dorn and Hart, Loxley, AL). Unstained (8 μm) sections were processed for dynamic histomorphometry using a UV inert mountant. Sections (5 μm) for static histomorphometry were stained with toluidine blue (Fisher) at pH 4.2. Microscopic analysis of all slides used an Olympus BX40 microscope computer-interfaced with the Osteomeasure system (Osteometrics, Atlanta, GA). Software calculations of various parameters are based on formulas defined by Parfitt [27]. Static parameters were measured on the trabecular bone immediately proximal to the growth plate, excluding endosteal surfaces. Field size for static parameters was 290 × 290 μm and the area measured under the growth plate extended 580 μm under the growth plate. Dynamic parameters were obtained using a Nikon Labophot scope equipped with epifluorescence to visualize the pulse-labeled calcein on trabecular surfaces. Mineralizing surface was calculated using single labels plus 1/2 double labeled surface. Field size for dynamic parameters was 350 × 350 μm and extended 700 μm under the growth plate.

2.8. Statistical analysis

All data are expressed as mean ± SEM. Bone turnover markers, urinary CCR, calciotropic hormones and gastrointestinal hormones were analyzed via repeated measures analysis of variance (ANOVA) and Tukey's studentized range for multiple comparisons test for pair-wise differences. All other parameters were analyzed via analysis of variance (ANOVA). Analyses were performed using SAS 9.4 (SAS Institute, Cary, NC). A two-sided $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of RYGB on weight and chow intake

Sham animals were ~40 g heavier at time of randomization than RYGB animals (371.3 ± 11.5 g versus 332.8 ± 10.9 g, Fig. 1B).

Weight change for RYGB (−11.5 ± 14.4 g, −3.6% total) and sham PF (−10.5 ± 21.0 g, −2.7% total) was significantly less than in sham AL (+42.6 ± 26.8 g, +12.1% total; $p = 0.0008$) throughout the study period. No significant difference in percentage weight change was seen between RYGB and sham PF ($p = 0.9334$). Mean weekly chow consumption was similar for all animals at baseline (191.3 ± 9.0 g/week). This decreased significantly in the post-operative period with sham AL rats returning to pre-operative consumption levels by post-op week 6 while RYGB animals returned by weeks 10–11 (Fig. 1C).

3.2. Biomarkers of bone turnover

Bone formation marker P1NP decreased across all four groups throughout the study with no statistical difference noted among groups by 10 or 14 weeks post-operatively. P1NP levels in RYGB groups were significantly higher than that in sham groups in week 6 (Fig. 2A). Both RYGB groups sustained mild elevations in serum OCN throughout the post-operative period of observation (Fig. 2B), while sham PF OCN slowly decreased. Baseline resorption marker CTX was ~60% greater in RYGB compared to sham PF rats (week 0). CTX levels were significantly higher in RYGB groups compared to sham groups at week 6 and week 14 (Fig. 2C). The R1000 and R5000 groups had similar patterns of change in serum OCN and CTX levels. The sham AL group was not sampled at week 0.

3.3. Measures of calcium metabolism and vitamin D

There were no differences in mean serum calcium, phosphate, or albumin across groups at end of the study (Fig. 2K–M). Urinary calcium excretion and CCR decreased markedly in RYGB groups post-operatively compared to sham (Fig. 2D). No statistical difference was noted between R1000 and R5000 groups over time, although a trend for higher calcium excretion was noted in R5000 group at week 14

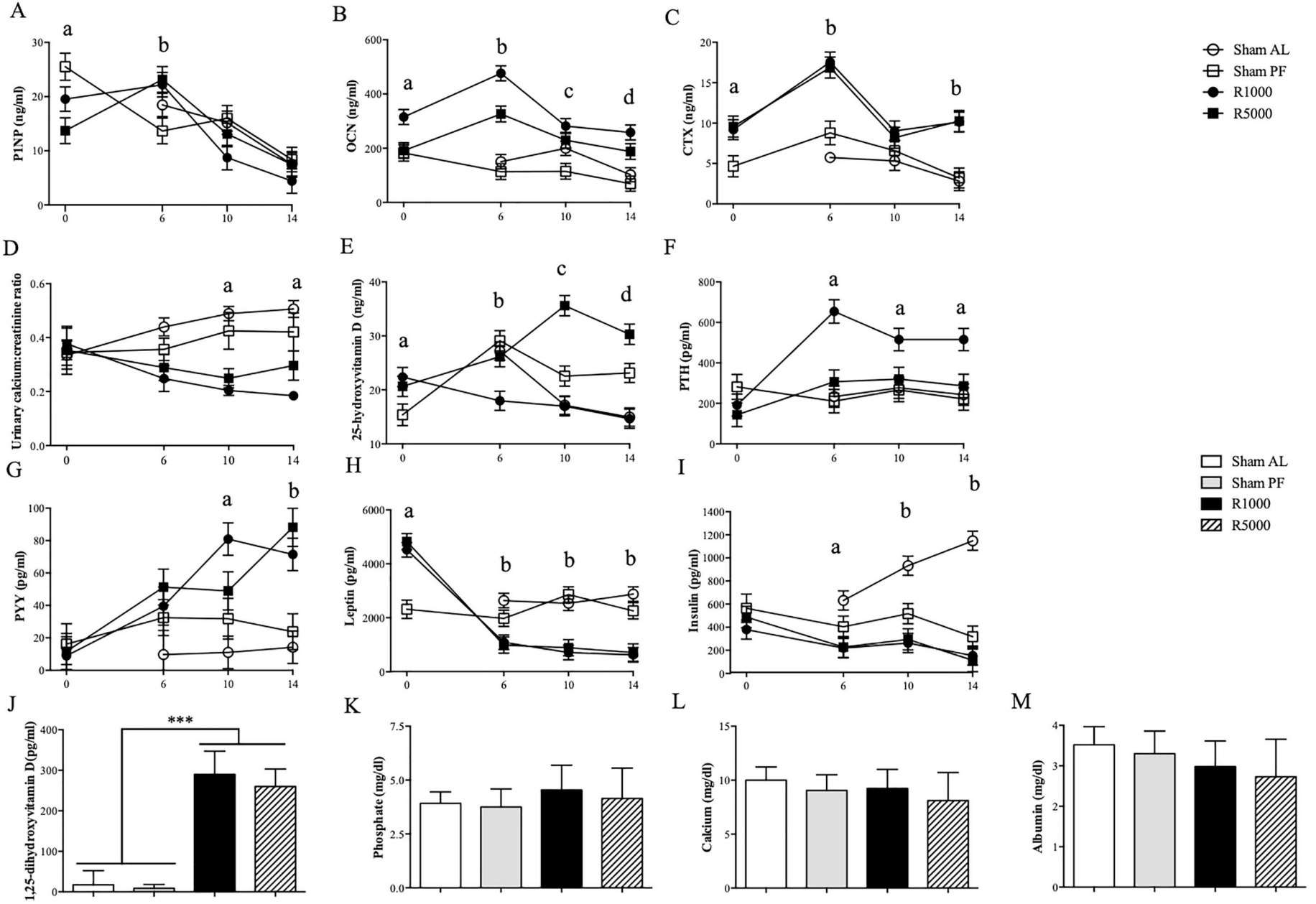

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Fig. 2. Biochemical assays. Serum was not collected in sham ad libitum group at week 0. (*A*) Serum P1NP levels. a, sham PF vs. R5000 $p < 0.05$. b, Sham vs. RYGB $p < 0.05$. (*B*) Serum OCN levels. a, R1000 vs. sham PF/R5000 $p < 0.01$. b, sham vs. R1000 $p < 0.001$; sham vs. R5000 $p < 0.001$; R1000 vs. R5000 $p < 0.001$. c, sham PF vs. R1000 $p < 0.001$. d, sham vs. RYGB $p < 0.001$. (*C*) Serum CTX levels. a, sham PF vs. RYGB $p < 0.05$. b, sham vs. RYGB $p < 0.001$. (*D*) a, sham vs. RYGB $p < 0.001$. (*E*) Serum 25-hydroxyvitamin D levels. a, sham PF vs. R1000/R5000 $p < 0.0001$. b, sham AL/sham PF/R5000 vs. R1000 $p < 0.001$. c, sham AL/sham PF/R1000 vs. R5000 $p < 0.001$. d, sham AL/R1000 vs. sham PF $p < 0.001$; sham AL/R1000 vs. R5000 $p < 0.001$; sham PF vs. R5000 $p < 0.001$ (*F*) Serum PTH levels. a, sham AL/sham PF/R5000 vs. R1000 $p < 0.001$. (*G*) Serum PYY levels. a, sham AL/sham PF vs. R1000 $p < 0.001$. b, sham vs. RYGB $p < 0.001$. (*H*) Serum leptin levels. a, sham PF vs. R1000/R5000 $p < 0.05$. b, sham vs. RYGB $p < 0.001$. (*I*) Serum insulin levels. a, sham AL vs. R1000/R5000 $p < 0.001$. b, sham AL vs. sham PF/R1000/R5000 $p < 0.001$. (*J*) Serum 1,25-dihydroxyvitamin D levels at week 14. ***Sham vs. RYGB $p < 0.001$. (*K*) Serum phosphate levels at week 14. (*L*) Serum calcium levels at week 14. (*M*) Serum albumin levels at week 14. Data presented as means ± SEM.

(Fig. 2D). The mean serum PTH level in the R1000 group was significantly elevated at week 6, 10 and 14 time-points (Fig. 2F) compared to baseline values and compared to sham PF and R5000 groups at the same time-points. Serum PTH levels in the R5000 group increased at week 6, 10 and 14 time-points compared to baseline but were not different than those in the sham PF group (Fig. 2F). Reflecting their greater level of supplementation, R5000 rats had markedly higher serum 25(OH)D levels at weeks 10 and 14 compared to R1000 group (Fig. 2E). Sham PF, at study end, had intermediate mean circulating 25(OH)D levels between the two RYGB groups (Fig. 2E). Serum calcitriol was more than five-fold greater in both RYGB groups than in the sham groups at week 14 (Fig. 2J), but there was no difference detected between the groups receiving two different levels of vitamin D supplementation.

3.4. Fat-secreted and gut hormones

Serum PYY increased in both RYGB groups throughout the study with marked increase at weeks 10 and 14 compared to the sham PF and AL groups (Fig. 2G), while serum leptin was markedly lower in RYGB animals compared to the sham groups at all post-operative time-points (Fig. 2H). Insulin levels in RYGB and sham PF rats were comparable (Fig. I), reflecting moderate weight loss among the groups, whereas the sham AL group showed increasing insulin levels coincident with their increasing weight, as expected.

3.5. μCT analysis and biomechanical testing

μCT analysis was performed in all groups. Analysis of cortical bone demonstrated significant effects of RYGB with reduced fractional cortical volume (Ct.BV/TV), thickness (Ct.Th.) and density (Ct. Density) in the RYGB groups (Table 1 and Fig. 3A, top panel) as well as an increased endocortical circumference. Periosteal circumference was comparable among all groups (Table 1). Analysis of trabecular bone parameters also revealed deficiencies in fractional bone volume (Tb. BV/TV), trabecular number (Tb.N.), thickness (Tb.Th.) and density (Tb. Density) in RYGB animals (Table 1 and Fig. 3A bottom panel). With respect to biomechanical testing, three-point bending indicated significant decreases in maximum load, fracture load and stiffness in femurs of RYGB animals, whereas the post-yield deflection values were greater in the RYGB groups indicating more malleable bone also results from the surgical intervention (Fig. 4A–C). Differences between the two sham groups, and between the two RYGB groups were not evident.

3.6. Histomorphometry

Histomorphometrical analysis confirmed the reduced trabecular bone volume fraction in RYGB animals, which had been identified by μCT (Fig. 3B, Table 1). In addition, osteoid volume was somewhat increased, and osteoblast counts expressed both per total bone area or per bone perimeter, were increased in RYGB rats compared to controls (Table 2). In contrast, osteoclast numbers were no different from control animals. Despite significant increases in osteoid volume, RYGB groups had a relatively greater mineral apposition rate in comparison to controls (Table 2).

4. Discussion

Using a well-established RYGB rat model with standardized light exposure and access to laboratory chow with customary (1000 IU) and enhanced (5000 IU) vitamin D content, we found that female RYGB animals on 5000 IU diet were able to achieve normal 25(OH)D levels in contrast to R1000 animals. Despite preventing vitamin D deficiency, both R5000 and R1000 groups had impressive cortical and trabecular bone loss associated with deterioration of biomechanics properties. The 5000 IU supplemented group demonstrated mild increases in serum PTH throughout the experiment as compared to baseline, together with significant decreases in 24-hour urinary calcium, and increased bone turnover marker levels.

In the RYGB procedure, a small gastric pouch is connected to the

Table 1
μCT data of rat femora at 14 weeks after RYGB or Sham procedures.

						p values
		Sham AL	Sham PF	R1000	R5000	Sham vs. RYGB
Cortical bone						
Ct.BV/TV	%	96.59 ± 0.80	96.74 ± 0.20	95.62 ± 0.70	95.84 ± 0.70	< **0.0001**
Ct.Th	mm	0.71 ± 0.05	0.72 ± 0.04	0.58 ± 0.05	0.60 ± 0.04	< **0.0001**
Ct.Density	mg/cm^3	1209.41 ± 12.04	1218.41 ± 14.68	1174.18 ± 50.57	1193.36 ± 32.08	**0.0038**
Ps.Pm	mm	10.27 ± 4.13	12.29 ± 0.48	12.32 ± 0.82	12.46 ± 0.74	0.1238
Ec.Pm	mm	5.79 ± 4.14	7.76 ± 0.49	8.70 ± 0.82	8.69 ± 0.86	**0.0109**
Trabecular bone						
Tb.BV/TV	%	32.30 ± 8.70	37.10 ± 9.90	19.40 ± 5.00	19.00 ± 5.50	< **0.0001**
Tb.N	mm^{-1}	4.97 ± 1.07	5.83 ± 1.46	2.99 ± 0.74	2.93 ± 1.00	< **0.0001**
Tb.Th	mm	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	< **0.0001**
Tb.Sp	mm	0.20 ± 0.05	0.17 ± 0.06	0.37 ± 0.14	0.39 ± 0.17	< **0.0001**
Tb.Density	mg/cm^3	919.34 ± 19.31	925.53 ± 15.14	869.48 ± 15.66	873.08 ± 13.26	< **0.0001**

Ct.BV/TV = cortical bone volume fraction; Ct.Th = average cortical thickness; Ps.Pm = periosteal perimeter; Ec.Pm = endocortical perimeter; Tb.BV/TV = trabecular bone volume fraction; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation. Values are mean ± SE. Bold *p* values are significant.

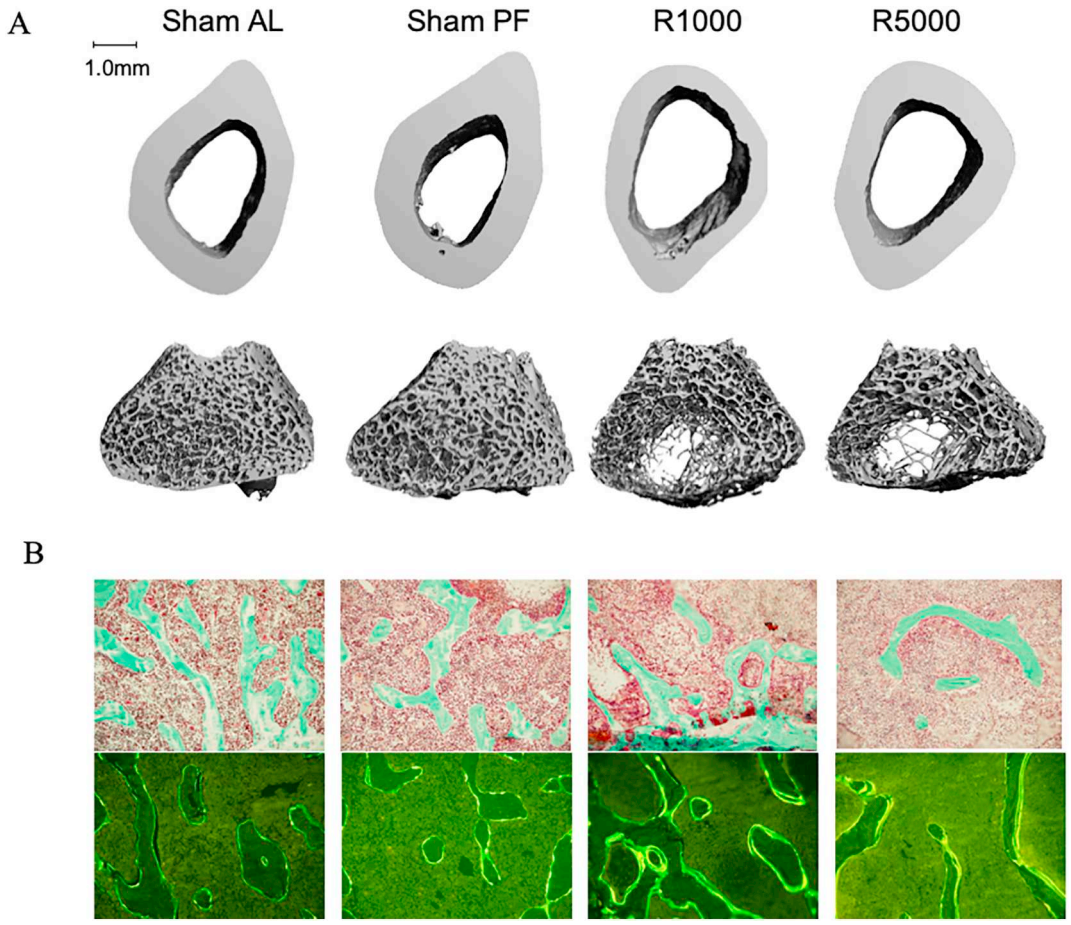

Fig. 3. μCT images and histology stains of rat femoral bone, 14 weeks after RYGB or sham operations. (*A*) Representative images of rat femoral cortical and trabecular bone. (*B*) Representative trichrome stains and calcein double labeling sections of rat femoral bone.

jejunum via the Roux limb, bypassing the remaining stomach, duodenum, and proximal small intestine thus reducing available intestinal absorptive surface for many micronutrients including vitamin D and calcium [28]. The resultant malabsorption of calcium would be expected to stimulate PTH secretion with consequent increases in circulating 1,25-dihydroxyvitamin D. Post-operative RYGB patients have

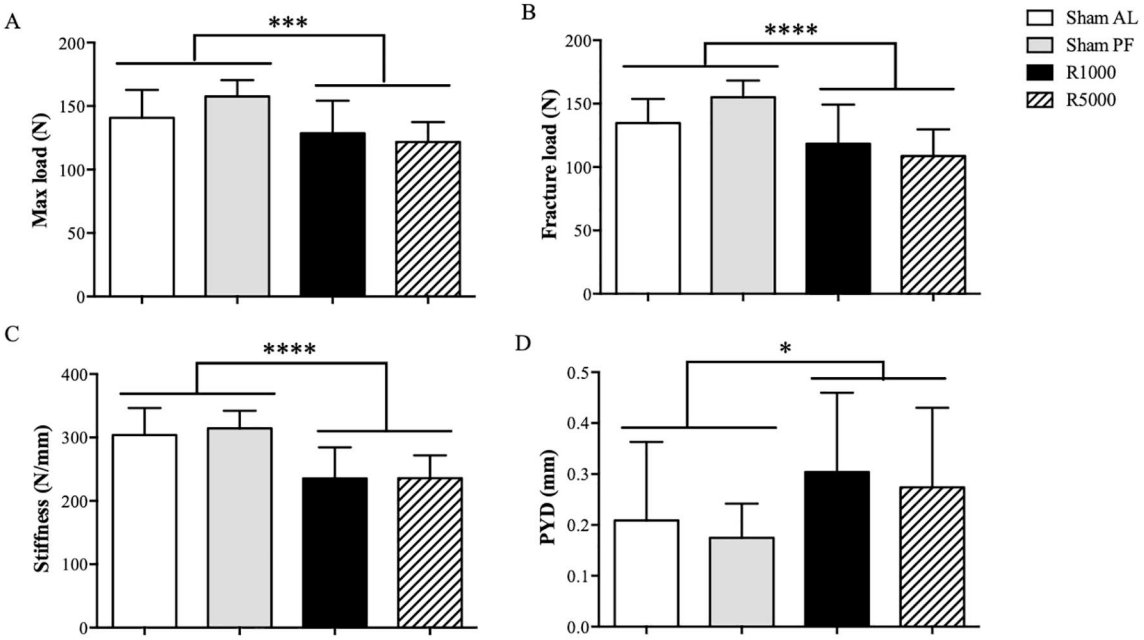

Fig. 4. Three-point bending test on rat femora at 14 weeks after RYGB or sham operations. (*A*) Maximum load. ***$p = 0.0009$. (*B*) Fracture load. ****$p < 0.0001$. (*C*) Cortical bone stiffness. ****$p < 0.0001$. (*D*) Post-yield displacement. *$p = 0.0302$. Data presented as means ± SEM.

Table 2
Histomorphometry data of rat femora at 14 weeks after RYGB or Sham procedures.

		Sham AL	Sham PF	R1000	R5000	Sham vs. RYGB
BV/TV	%	35.000 ± 10.390	40.440 ± 10.460	21.010 ± 5.631	19.620 ± 6.547	< **0.0001**
OV/TV	%	0.303 ± 0.132	0.251 ± 0.123	0.466 ± 0.138	0.420 ± 0.117	< **0.0001**
NOb/TAR	mm^{-2}	15.580 ± 9.427	13.100 ± 11.470	36.190 ± 14.410	28.560 ± 14.220	< **0.0001**
NOb/BPm	mm^{-1}	1.623 ± 0.818	1.239 ± 0.965	5.659 ± 2.227	4.424 ± 1.832	< **0.0001**
NOc/TAR	mm^{-2}	8.002 ± 4.885	7.381 ± 2.409	6.640 ± 4.921	5.157 ± 2.195	0.1741
NOc/BPm	mm^{-1}	0.822 ± 0.437	0.727 ± 0.172	1.020 ± 0.711	0.804 ± 0.288	0.3913
MAR	μm/day	1.231 ± 0.261	1.051 ± 0.166	1.797 ± 0.167	1.556 ± 0.258	< **0.0001**

BV/TV = bone volume fraction; OV/TV = osteoid volume fraction; Nob = number of osteoblasts; TAR = tissue area; NOc = number of osteoclasts; BPm = bone perimeter; MAR = mineral apposition rate. Values are mean ± SE. Bold *p* values are significant.

been shown to have reduced intestinal calcium absorption even with optimization of vitamin D [29], consistent with the finding of reduced urinary calcium excretion that we observed in our RYGB animals. Despite normal serum calcium levels, both RYGB animal groups were calcium depleted, reflected in their significantly decreased urinary calcium excretion at 6, 10 and 14 week time-points. Circulating 1,25-dihydroxyvitamin D was comparable between our two RYGB groups and > 5-fold higher than in control animals. The marked increase in circulating 1,25-dihydroxyvitamin D levels in R5000 animals despite modest elevations in serum PTH and normal serum phosphate levels is not entirely explained, but suggests that vitamin D-independent reductions in calcium absorption may be at work. Stemmer et al. (2013) saw similar calcium and vitamin D trends in post-RYGB male rats and attempted to correct this using a "rescue diet" – an isocaloric HFD modified by the addition of 2200-IU vitamin D per kg, 2% calcium – for 8 weeks. This diet resulted in an overall increase of serum 25-hydroxyvitamin D level and a normalization of serum calcium levels in their RYGB rats, but the detrimental effect of RYGB on bone did not reverse [23]. Our RYGB animals with vitamin D supplementation also had 2% calcium (roughly twice laboratory rat nutrient requirements) for a total of 12 weeks – yet the bone phenotype did not change.

If a primary driver of bone loss in the RYGB population is impaired intestinal calcium absorption, one might expect human RYGB bone histomorphometry to be reflective more of osteomalacia than osteoporosis. However, the true bone loss phenotype that occurs after RYGB is not known because no iliac crest biopsy study has been published in RYGB patients. Therefore, the best surrogates are iliac crest biopsy studies done in other bariatric populations. Two such studies were performed in 74 different biliopancreatic diversion/duodenal switch patients, with bone biopsies done before surgery and mean of 41 months post-operatively. In this population with normal 25-OH vitamin D levels, iliac crest biopsy results were very similar to bone histomorphometry in our model – increased osteoid volume, markedly decreased cortical thickness, and increased bone formative rate/bone surface [30,31]. Therefore, the bone phenotype in our model is consistent with that of the human condition. The histomorphometry findings identified increases in osteoid parameters in the RYGB groups, a hallmark of osteomalacia. However, the dynamic histomorphometry following calcein labeling showed an increase in the mineral apposition rate (Table 2), inconsistent with the delay in mineralization seen in osteomalacia. The finding could represent a robust anabolic compensatory response to the early rapid bone loss following the surgical procedure and feeding compromise. Such an interpretation is supported by the increased osteoblast numbers in RYGB animals. Although these results extend our current understanding of the skeletal effects of RYGB, the mechanism is still not fully understood. It would be of great interest to extend these experiments for longer follow-up periods to identify whether complete recovery of skeletal deficiencies is corrected.

Patients with obesity have a high prevalence of vitamin D deficiency, even before their bariatric procedures [32]. Therefore, RYGB patients are already at high risk of vitamin D deficiency, even before they undergo surgery and develop malabsorption of fat-soluble vitamins [33,34]. Despite a lack of consensus regarding the optimal supplementation regimen, universal vitamin D supplementation has been recommended for all patients undergoing RYGB [35]. These recommendations have been corroborated by studies demonstrating that adequate vitamin D supplementation corrects serum 25-hydroxyvitamin D in RYGB patients [12,36,37]. Luger et al. (2016) performed a double-blind randomized placebo-controlled trial on patients after bariatric surgery [38]. After 6 months of vitamin D supplementation, the intervention group had higher serum 25-hydroxyvitamin D and less secondary HPT compared with a control group [38]. Overall, vitamin D supplementation remains an important component of post-RYGB nutrition, but our data suggests skeletal effects can still occur despite supplementation.

The finding of increased PTH levels in many human patients after RYGB has led to the assumption that PTH-induced bone resorption is the main driver of bone loss. Findings from this animal model suggest that bone defects may occur with limited changes in serum PTH [24] or persist afterwards despite correction of circulating PTH to levels comparable to control rats [23,39]. These data suggest that PTH-induced bone resorption is not the sole cause of RYGB-related bone loss. We found persistent elevations in osteocalcin and CTX (resorptive marker), consistent with a high bone turnover state reported following RYGB surgery in humans [40,41] and rats [22–24,42].

P1NP levels in our RYGB animals were elevated only at week 6 and decreased consistently after that time-point. One possible explanation for this finding is that PYY, a gut-derived satiety peptide of the neuropeptide Y family, has been found to be negatively correlated with the bone formation marker PINP [43]. In our study, the increase in RYGB circulating PYY, previously described in both animal and human studies [22,43,44], may have contributed to the decrease in P1NP levels. Finally, leptin, a fat secretory hormone known to effect bone remodeling, was lower in RYGB animals versus shams. Leptin is positively associated with bone mineral density [42], raising the possibility that lower serum leptin may also influence bone resorption markers [36,45,46].

5. Conclusion

Clinical efforts after RYGB should emphasize peri-operative calcium supplementation strategies along with appropriate vitamin D supplementation, but in our female rat model, RYGB-associated bone loss is not rescued by providing sufficient dietary vitamin D to correct circulating 25-hydroxyvitamin D levels. The etiology of this bone disease does not appear to be entirely driven by PTH-induced bone resorption. Instead, the RYGB-associated bone phenotype in this model is likely multi-factorial in origin and may include nutrient malabsorption (particularly calcium) as well as altered fat and/or gastrointestinal hormones.

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CRediT authorship contribution statement

Aidi Niu: Formal analysis, Writing - original draft, Visualization. **Thomas O. Carpenter**: Resources, Writing - review & editing, Supervision. **Jayleen M. Grams**: Writing - review & editing, Visualization, Supervision. **Shahab Bozorgmehri**: Software, Formal analysis, Data curation. **Steven M. Tommasini**: Software, Formal analysis. **Anne L. Schafer**: Writing - review & editing, Supervision. **Benjamin K. Canales**: Conceptualization, Methodology, Resources, Investigation, Writing - review & editing, Supervision, Visualization, Funding acquisition.

Declaration of Competing Interest

All authors state that they have no conflicts of interest.

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