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Decreased Bone Mineral Density and Reduced Bone Quality in H^+/K^+ATPase Beta-Subunit Deficient Mice

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ABSTRACT

Proton pump inhibitors (PPIs) are widely used against gastroesophageal reflux disease. Recent epidemiological studies suggest that PPI users have an increased risk of fractures, but a causal relationship has been questioned. We have therefore investigated the skeletal phenotype in H^+/K^+ATPase beta-subunit knockout (KO) female mice. Skeletal parameters were determined in 6- and 20-month-old KO mice and in wild-type controls (WT). Whole body bone mineral density (BMD) and bone mineral content (BMC) were measured by dual energy X-ray absorptiometry (DXA). Femurs were examined with μCT analyses and break force were examined by a three-point bending test. Plasma levels of gastrin, RANKL, OPG, osteocalcin, leptin, and PTH were analyzed. KO mice had lower whole body BMC at 6 months (0.53 vs. 0.59 g, $P = 0.035$) and at 20 months (0.49 vs. 0.74 g, $P < 0.01$) compared to WT as well as lower BMD at 6 months (0.068 vs. 0.072 g/cm^2, $P = 0.026$) and 20 months (0.067 vs. 0.077 g/cm^2, $P < 0.01$). Mechanical strength was lower in KO mice at the age of 20 months (6.7 vs. 17.9 N, $P < 0.01$). Cortical thickness at 20 months and trabecular bone volume% at 6 months were significantly reduced in KO mice. Plasma OPG/RANKL ratio and PTH was increased in KO mice compared to controls. H^+/K^+ATPase beta subunit KO mice had decreased BMC and BMD, reduced cortical thickness and inferior mechanical bone strength. Whereas the mechanism is uncertain, these findings suggest a causal relationship between long-term PPI use and an increased risk of fractures. J. Cell. Biochem. 113: 141–147, 2012.

KEY WORDS: PROTON PUMP INHIBITORS; OSTEOPOROSIS; PARATHYROID HORMONE

Proton pump inhibitors (PPIs) are widely used in the management of acid-related disease such as gastroesophageal reflux, and many persons use potent inhibitors of gastric acid secretion for the relief of symptoms. However, the consequences of long-term acid inhibition are not fully known. Whereas short-term treatment of children with PPI has not been found to affect parameters of bone mineralization [Kocsis et al., 2002], two large case control studies strongly suggest that patients using PPIs have an increased risk of hip fracture [Yang et al., 2006]. More recently, others have found an association between long-term PPI use and osteoporosis-related fractures [Targownik et al., 2008] and vertebral fractures in particular [Roux et al., 2009]. However, PPI use has so far not been shown to be associated with low bone mineral density (BMD) or increased rate of bone loss [Targownik et al., 2010].

A causal relationship between PPI use and the observed increase in fracture risk has been doubted for several reasons [Laine, 2009]. Prospective studies in patients using long-term PPIs have not been published, there has been little experimental research supporting such findings, and a certain mechanism has not been established.

The gastric proton pump, H^+/K^+-adenosin triphosphase (H^+/K^+ATPase), is a heterodimeric protein consisting of an α- and a β-subunit encoded by the genes *Atp4a* and *Atp4b*, respectively, in both men and mice [Read et al., 2007]. Homozygote gastric H^+/K^+ATPase β-subunit KO mice with deficient gastric acid secretion and increased gastrin levels were generated previously by Scarff et al. [1999]. The effects induced are the same as observed by long-term PPI-use. Gastric H^+/K^+ATPase is expressed only in scarce amounts outside the oxyntic gastric mucosa, and it has not been

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found in bone [Herrmann et al., 2007]. However, there are in vitro experiments suggesting that the PPI omeprazole decreases bone resorption [Tuukkanen and Vaananen, 1986] possibly through inhibition of the osteoclastic vacuolar H^+/K^+ATPase [Mizunashi et al., 1993]. PPI has also been added to cement used in orthopedic surgery and delivered in high concentrations locally it has a positive effect on bone formation [Sheraly et al., 2009]. On the other hand, we have previously seen that rats given a PPI for 3 months have reduced bone mineralization [Cui et al., 2001] suggesting that systemic PPI administration does not prevent bone resorption in vivo.

In the present study, we have examined the skeletal phenotype of the gastric proton pump β-subunit KO mice.

MATERIALS AND METHODS

ANIMALS

H^+/K^+ATPase KO mice originally had a BalbC/B6 genetic background [Scarff et al., 1999; Franic et al., 2001], later BalbC (CrSk) [Tennant et al., 2008]. In this study, we used wild-type (WT) BalbC (Møllegård, DK) as controls. For the experiments, we used female KO mice 6 months old (n = 7) and age-matched WT (n = 13) as well as KO mice 20 months old (n = 7) and age-matched controls (n = 5). All mice were housed in wire-top cages with aspen woodchip bedding from B&K Universal Ltd. Room temperature was 24 ± 1°C with a relative humidity of 40–50% and a 12-h light/dark cycle. The Rat and Mouse Diet of B&K and tap water were provided ad libitum. Animals were sacrificed by exsanguination after blood sampling from the inferior caval vein while anesthetized with 4 ml/kg body weight of a combination of haloperidol (1.65 mg/ml), fentanyl (0.25 mg/ml), and midazolam (2.5 mg/ml). The study was approved by the Animal Welfare Committee at St Olav's Hospital, Trondheim, Norway.

DUAL X-RAY ABSORPTIOMETRY (DXA) MEASUREMENTS

DXA measurements were performed using a Hologic QDR 4500A and small animal software. Body weight (g), fat mass (g), and lean mass (g), bone mineral content (BMC) (g), and (BMD) (g/cm^2) in whole body, were measured in duplicates by DXA. The animals were anesthetized with 4 ml/kg body weight of a combination of haloperidol (1.65 mg/ml), fentanyl (0.25 mg/ml), and midazolam (2.5 mg/ml) before the procedure.

MICRO-COMPUTED TOMOGRAPHY (μCT) MEASUREMENTS

The left femurs were dissected from euthanized animals, the lengths were measured, and they were stored in 4% phosphate-buffered formaldehyde at −20°C until analyses by μCT using Skyscan 1172 (Skyscan, Kontich, Belgium) The samples were scanned with a voxel size of 8 μm and reconstructed by use of a manufacturer-provided software (NRecon, SkyScan). Three areas of 1 mm in length were analyzed: proximal diaphysis (slices taken at the level of 0.5 mm from bottom of third trochanter), mid-diaphysis, and distal-diaphysis. The different sections were separated by 2.5 mm. Cortical volumetric bone mineral density (vBMD) and three-dimensional parameters of cortical and trabecular bone were calculated with the Skyscan program CTAn (version 1.10.0.1 for all parameters except cortical thickness: version 1.9.00).

For vBMD measurements and 3D analyses of cortical bone, a cubic volume of 0.0128 mm^3 was examined for mid- and proximal sections. For 3D analyses of trabecular bone, a volume of 0.447 μm^3 of the distal sections was analyzed. To determine vBMD values, a threshold of 1–255 was applied to the volumes of interest. The 3D parameters of cortical and trabecular bone were calculated after applying a threshold of 86–255 so that only fully mineralized bone tissue was analyzed.

THREE-POINT BENDING TESTS

The right femurs were dissected and stored at −70 °C until the three-point bending test was performed using a Zwicki (Zwick Roell, Ulm, Germany) with a 200 N load cell. The right femur was used with the anterior surface facing up during the three-point bending test. The ultimate stress σ was calculated with the formula [Jamsa et al., 1998].

$$\sigma = \frac{FLc}{4I}$$

F: force at failure; L: distance of lower supports (6 mm); c: half diameter of bone in direction of break force; I: moment of inertia. C and I were measured on one slice in the region of failure (mid shaft) with the program CTAn 1.9.0.0 with a threshold of 86–255.

RNA ISOLATION AND cDNA SYNTHESIS

Left tibia samples were crushed in a mortar on liquid nitrogen and added TRIzol® Reagent (Invitrogen, Norway), while frozen samples from corpus were homogenized in TRIzol® Reagent with a knife-rotor homogenizator (ULTRA-TURRAX T25, JANKE & KUNKEL, IKA®-Labortechnik, Germany), before RNA was isolated according to the manufacture's protocol. RNA-containing solution was applied directly to obtain a first-strand complementary DNA (cDNA) using the iScript cDNA Synthesis Kit with oligo(dT) primers (Bio-Rad, CA).

REAL-TIME PCR QUANTIFICATION

Reactions were performed using 2× iQ SYBR Green Supermix (Bio-Rad) and monitored using Stratagene's Mx3000P real-time PCR system and 2X iQ SYBR Green Supermix (Bio-Rad). cDNA samples were analyzed in triplets for genes of interest and reference gene (β-actin). β-actin RT-PCR was used to monitor RNA integrity and for normalization. The C_t value was measured for each sample, and arbitrary units were calculated using standard curves that consisted of serial dilutions of cDNA. Contamination by genomic DNA was ruled out by performing PCR analysis where the RT-enzyme had been omitted in the cDNA synthesis. Specificity of each primer pair was confirmed by melting curve analysis, and data were calculated from standard curves and related to a housekeeping gene. Table I shows the intron-spanning primer sequences.

PLASMA GASTRIN, OSTEOPROTEGERIN (OPG), RECEPTOR ACTIVATOR OF NUCLEAR κB LIGAND (RANKL), OSTEOCALCIN, LEPTIN, AND PARATHYROID HORMONE (PTH) ANALYSES

At termination of the study, blood was drawn from the inferior caval vein, and plasma was frozen at −20°C until analyses were

TABLE I. Primers Used in Real-Time PCR Quantification

Gene	Primer sequences	Species	Amplicon size (bp)	Gene bank accession number
Atp4b	S 5′-GGCCTCACACAGAGGAGACT-3′ AS 5′-AGATGCACAAGGCAAAGAGC-3′	Mouse	250	NM_09724.2
Atp6v1a	S 5′-TCGGAAACCCTGAGAGAGAA-3′ AS 5′-ATACCCAGCGTTGCAGAAGT-3′	Mouse	100	NM_007508.5
Atp6v1b2	S 5′-GCTGCTGGATTACCACACAA-3′ AS 5′-CTCCCATAGCAGCAAACACA-3′	Mouse	127	NM_007509.2
β-actin	S 5′-CTGGCTCCTAGCACCATGA-3′ AS 5′-AGGCACCAATCCACACAGA-3′	Mouse	73	NM_031144.2

performed. Gastrin was measured by RIA as previously described [Kleveland et al., 1985]. OPG, RANKL, osteocalcin, and leptin concentrations in plasma were analyzed by enzyme-linked immunosorbent assays (ELISA) using multianalyte profiling (Luminex-100; Luminex Corporation, Austin, TX). The intraassay CVs were RANKL 3.1%; OPG 3.1%; leptin 3.7%; osteocalcin 2.3%; IL-6 3.7%. Plasma PTH was analyzed with an ELISA method (Immutopics, Inc., San Clemente, CA), only in mice at age 6 months.

STATISTICS

Measured values are presented as mean ± 1 standard deviation (SD). Differences between the two groups were evaluated using an unpaired, two-tailed Student *t*-test when comparing data with normal distribution (analyzed with Kolmogorow–Smirnov (KS)–normality test). $P < 0.05$ was considered significant.

RESULTS

BODY COMPOSITION AND FEMUR LENGTHS

The data are presented in Table II. Neither total body mass nor lean body mass differed significantly between the groups at 6 and 20 months compared to controls. However, H^+/K^+ATPase KO mice had less body fat (g) at 6 months (4.91 ± 1.4 g vs. 3.68 ± 1.0 g, $P = 0.012$) as well as at 20 months (3.12 ± 0.6 vs. 4.45 ± 1.0 g, $P = 0.022$) compared to controls.

The body fat in % was significantly lower in H^+/K^+ATPase KO mice at 6 months (14.4 ± 3.7 vs. 21.1 ± 4.5, $p = 0.012$) as well as at 20 months compared to WT controls (11.6 ± 2.1 vs. 15.3 ± 2.1, $P = 0.017$). Femur lengths did not differ between the groups.

TABLE II. Body Composition and Femur Length in 6- and 20-Month-Old H^+/K^+ATPase Beta Subunit Knockout (KO) and Control Mice

	6 mo control	6 mo KO	20 mo control	20 mo KO
Body weight (g)	23.1 ± 2.88	25.5 ± 1.31	28.4 ± 3.85	26.6 ± 2.08
Fat (g)	4.91 ± 1.37	3.68 ± 0.96	4.45 ± 0.96	3.12 ± 0.63
Fat (%)	21.1 ± 4.48	14.4 ± 3.67*	15.3 ± 2.11	11.6 ± 2.11*
Lean mass (g)	18.3 ± 1.98	21.9 ± 1.41	23.4 ± 1.81	23.2 ± 2.77
Femur length (mm)	15.3 ± 0.74	15.3 ± 0.67	16.8 ± 0.73	16.2 ± 0.27

*$P < 0.05$ significantly different compared to controls.

WHOLE BODY BMC AND BMD EVALUATED BY DXA

H^+/K^+ATPase KO mice had lower whole body BMC (0.53 ± 0.02 vs. 0.59 ± 0.07 g, $P = 0.035$) and BMD (0.0068 ± 0.003 vs. 0.072 ± 0.004 g/cm^2, $P = 0.026$) at 6 months as well as at 20 months compared to WT controls (BMC 0.49 ±0.097 vs. 0.74 ± 0.075 g, $P < 0.01$ and BMD 0.067 ± 0.005 vs. 0.077 ± 0.002 g/cm^2, $P < 0.01$) (Fig. 1).

BONE ARCHITECTURE OF THE FEMUR EVALUATED BY μCT

Femurs of H^+/K^+ATPase KO mice had lower cortical thickness at 20 months (Table III). At 6 months H^+/K^+ATPase KO mice had lower trabecular bone volume%, lower trabecular number and longer distance between the trabeculae (Table III). Other differences between the groups did not reach a significant level.

MECHANICAL PROPERTIES OF THE FEMUR

Break force and calculated ultimate stress σ are presented in Figure 2. H^+/K^+ATPase KO mice had femurs with lower break force (6.8 ± 4.8 N vs. 17.9 ± 6.6 N, $P < 0.01$) as well as lower calculated ultimate stress σ (1.48 ± 1.2 MPa vs. 5.76 ± 2.6 MPa, $P < 0.01$) compared to controls at 20 months, whereas the mechanical properties of the femur did not differ significantly at 6 months.

RELATIVE GENE EXPRESSION IN CORPUS AND TIBIA

There was, as expected, no detectable relative gene expression of the gastric H^+/K^+ATPase β-subunit gene *Atp4b* in the corpus tissue from KO mice, while expressed in corpus from all controls (data not shown). Neither controls nor KO mice had any detectable expression of *Atp4b* in bone tissue (tibia). To examine whether gene expression of the osteoclastic vacuolar proton pump subunits α and 2β were affected in KO mice compared to controls, the relative expression of *Atp6v1a* and *Atp6v1b2* in corpus and bone tissue (tibia) were examined, but no significant differences between the groups were detected (data not shown).

PLASMA GASTRIN, OPG, RANKL, OSTEOCALCIN, LEPTIN, AND PTH LEVELS

The detailed results are presented in Figure 3. Plasma gastrin was significantly higher in H^+/K^+ATPase KO mice than in controls at 6 months (71 ± 6 pM vs. 14 ± 3 pM, $P < 0.01$) and 20 months

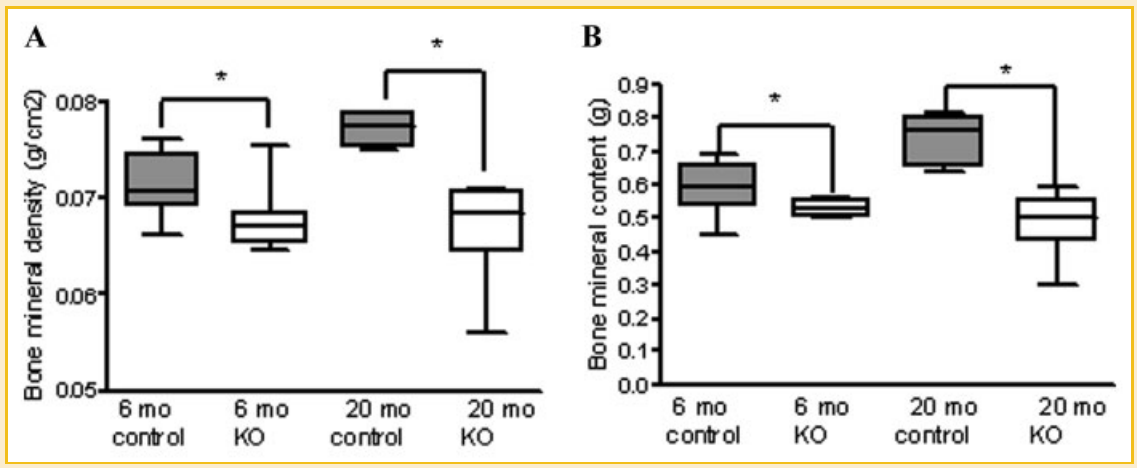

Fig. 1. Whole body bone mineral density (BMD) (A) and bone mineral content (BMC) (B) in 6- and 20-month-old H^+/K^+ATPase beta subunit knockout (KO) and control mice. *P-value < 0.05 compared to age-matched controls.

(47 ± 7 pM vs. 11 ± 1 pM, $P < 0.01$). Plasma RANKL was lower at 6 months (90 ± 6 pg/mL vs. 56 ± 7 pg/mL, $P < 0.01$), but not at 20 months, whereas OPG was higher in KO mice at both ages. The OPG/RANKL ratio was significantly higher in KO mice compared to controls at 6 months (1.58 ± 0.06 vs. 0.68 ± 0.04, $P < 0.01$), but did not reach significance at 20 months (1.45 ± 0.24 vs. 0.85 ± 0.05, $P = 0.067$).

The osteocalcin levels did not differ between the groups. KO mice had lower plasma leptin levels compared to controls at 6 months (355 ± 46 pg/μL vs.735 ± 143 pg/μL, $P < 0.05$), but not at 20 months.

PTH levels were significantly higher in KO mice than in controls (236 ± 26 pg/mL vs. 104 ± 18 pg/mL, $P < 0.01$) (only analyzed at 6 months).

DISCUSSION

Several large case control studies have demonstrated an association between PPI use and increased risk of hip fractures [Yang et al., 2006] osteoporotic fractures overall [Vestergaard et al., 2006; Targownik et al., 2008; Gray et al., 2010] and spine fractures in particular [Roux et al., 2009]. In a recent metaanalysis, spine fractures seem most consistently associated with PPI use, but not with histamine-2 receptor antagonists [Kwok et al., 2010]. Few animal models for studying changes in bone in subjects with gastric hypoacidity have so far been used, but we have previously described that omeprazole given to young male rats for 3 months negatively affects whole body BMD [Cui et al., 2001].

In this study, we describe the bone phenotype in H^+/K^+ATPase KO mice, which have gastric hypoacidity and represent a model of long-term proton pump inhibition. We found that both 6- and 20-month-old KO mice have an osteoporotic phenotype with decreased total body BMC and BMD in spite of no differences in body weight or lean mass. However, KO mice at the age of six months had a significantly decrease in body fat% compared to controls. Twenty-month-old KO mice exhibited lower femoral cortical thickness and, in 6-month-old mice, a lower trabecular bone volume compared to age-matched controls and inferior mechanical properties with a lower break force and lower stress σ values were determined.

The group that generated and donated the HKATPase KO mice used a BalbC strain as controls in their later experiments [Tennant et al., 2008], while we used BalbC mice from a different producer as controls. A difference in genetic background may therefore

TABLE III. Bone Architecture Parameters of the Femur Metaphysic in 6- and 20-Month-Old H^+/K^+ATPase Beta Subunit Knockout (KO) and Control Mice

	6 mo control	6 mo KO	20 mo control	20 mo KO
Cortical bone volume (%)	99.78 ± 0.000	98.96 ± 1.72	96.43 ± 0.116	94.65 ± 3.19
Cortical thickness (mm)	0.249 ± 0.027	0.25 ± 0.012	0.259 ± 0.014	$0.18 \pm 0.006^{**}$
Cortical BMD (g/cm^3)	1.82 ± 0.1	1.74 ± 0.07	1.85 ± 0.03	1.76 ± 0.13
Trabecular bone volume (%)	8.15 ± 3.97	$3.34 \pm 1.7^{**}$	0.198 ± 0.114	0.284 ± 0.21
Trabecular thickness (mm)	0.052 ± 0.006	0.047 ± 0.008	0.039 ± 0.005	0.033 ± 0.017
Trabecular number (1/mm)	0.971 ± 0.469	$0.455 \pm 0.24^{*}$	0.028 ± 0.012	0.043 ± 0.027
Trabecular separation (mm)	0.26 ± 0.045	$0.378 \pm 0.079^{**}$	0.566 ± 0.057	0.515 ± 0.087

Mo, months age; vBMD, volumetric bone mineral density.
*$P < 0.05$;
**$P < 0.01$ significantly different compared to controls.

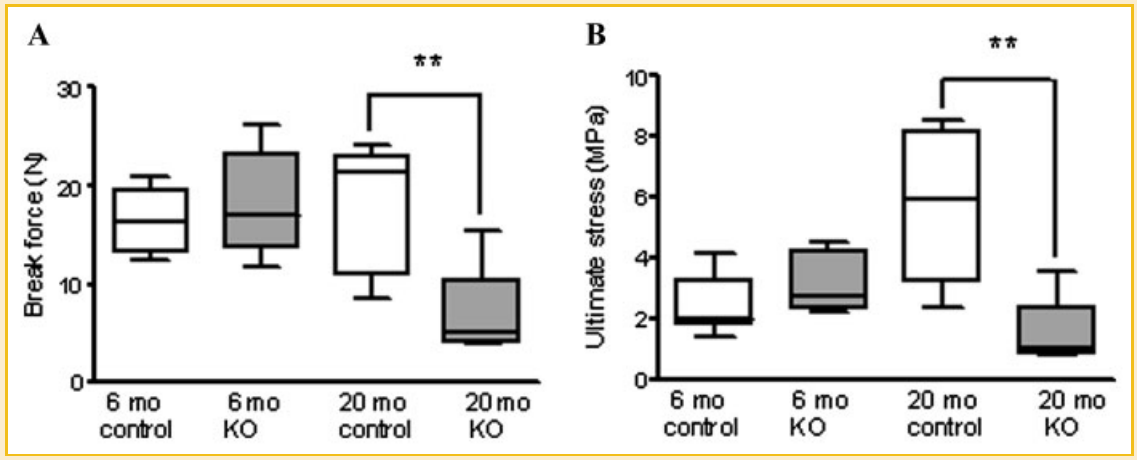

Fig. 2. Break force (A) and calculated ultimate stress σ (B) in 6- and 20-month-old H^+/K^+ATPase beta subunit knockout (KO) and control mice. *P-value < 0.05, **P-value < 0.01 compared to age-matched controls.

influence the results. The skeletal differences between the groups observed at 20 months are, however, so pronounced that is unlikely that this is due to minor differences in genetic background.

Our results confirmed the assumption of no expression of the gastric H^+/K^+ATPase pump in osteoclasts in bone, as no expression of *Atp4b* was detected in bone tissue from either KO mice or controls. The relative gene expression of the osteoclastic vacuolar V-H^+/K^+ATPase α- or 2β-subunits was not found to be affected in neither gastric mucosa of corpus nor bone tissue, indicating that gene activation of the osteoclastic proton pump is not involved in the observed skeletal effects.

H^+/K^+ATPase KO mice had a decrease in plasma leptin levels. This might be explained by the decrease in body fat%, as amount of adipose tissue is the strongest determinant of plasma leptin levels. Leptin has emerged as a significant factor in the regulation of bone mass [Reseland et al., 2001; Reid et al., 2006]. We have previously shown that leptin is expressed in bone cells and that it stimulates osteoblast differentiation and mineralization [Reseland et al., 2001]. Furthermore, an inhibitory effect on bone resorption has been demonstrated [Holloway et al., 2002]. Thus, low leptin levels may contribute to the observed decrease in bone mass and deterioration of bone architecture.

The H^+/K^+ATPase KO mice have gastric hypoacidity and secondary hypergastrinemia, which is also seen in PPI users as well as patients with abolished gastric acid secretion due to chronic atrophic gastritis/pernicious anemia. In a previous study, we found that rats receiving omeprazole also had increased plasma level of both gastrin and histamine [Cui et al., 2001]. There are indications that histamine participates in the regulation of bone resorption, as histidine decarboxylase deficient mice have an increased BMD.

Patients with pernicious anemia have lower BMD [Eastell et al., 1992] and an increased risk of osteoporosis related fractures [Goerss et al., 1992; Merriman et al., 2010] suggesting that gastric anacidity or hypergastrinemia, which are the common factors in the two groups of patients, affect BMD and strength. In a recent cross-sectional study, however, no differences in BMD were observed between patients with autoimmune gastritis and controls [Kakehasi et al., 2009].

Recently it was found that mice deficient in CCKB receptors, analogous to the gastrin receptor in humans, have gastric hypoacidity and develop hypocalcemia, hyperparathyroidism, and osteoporosis [Schinke et al., 2009]. These changes could be reversed by calcium supplementation, indicating that alterations in calcium homeostasis can be driven by defects in gastric acidification [Schinke et al., 2009].

There are no long-term studies on the effects of PPIs on calcium absorption. Previous short-term experiments investigating the effect of gastric acidity on calcium absorption, have suggested that reduced calcium absorption could explain increased fracture risk observed in patients. PPIs decrease calcium absorption in rats [Chonan et al., 1998] as well as in women using PPI [O'Connell et al., 2005]. In patients with pernicious anemia some have found calcium absorption to be lower than in controls [Recker, 1985], whereas others have found no difference [Eastell et al., 1992]. An increased fracture risk in PPI users, but only among those not taking calcium supplements has also been reported [Yu et al., 2008]. Secondary hyperparathyroidism may be the causative mechanism explaining the increased fracture risk in PPI users. Both hypoacidic CCKB receptor deficient mice [Schinke et al., 2009] as well as H^+/K^+ATPase KO mice have higher PTH levels than controls, whereas reports on secondary hyperparathyroidism in human PPI users are lacking. Elevation of circulating PTH levels is known to result in increased bone resorption through stimulation of RANKL and inhibition of OPG from osteoblasts [Huang et al., 2004]. In contrast to this, we observed a rise in the OPG/RANKL ratio in plasma in the H^+/K^+ATPase KO mice. This might be a compensatory response to increased bone resorption, but might also reflect that OPG and RANKL are derived from extraskeletal sources. We could not detect any differences in the bone formation marker osteocalcin between the groups.

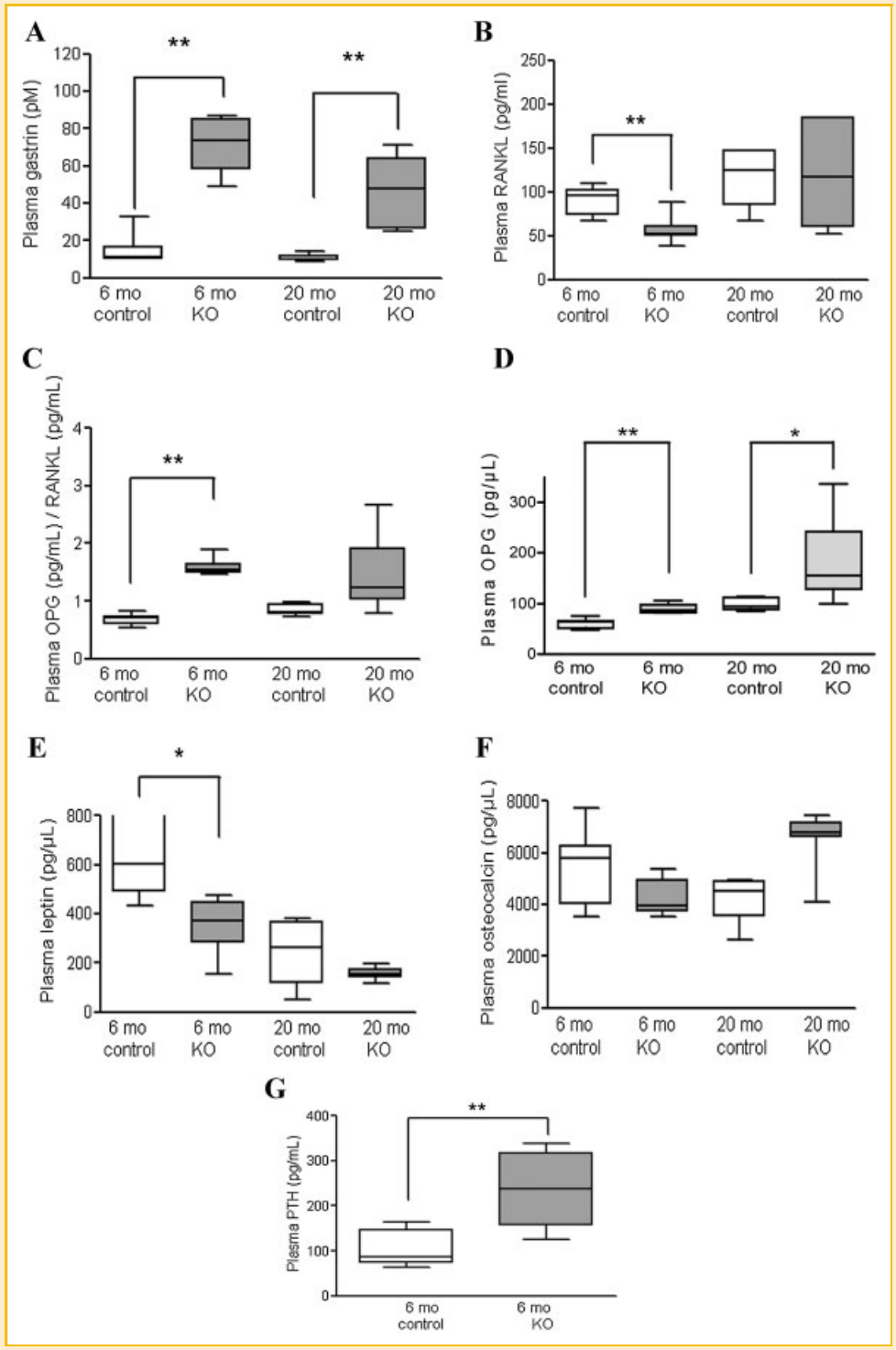

Fig. 3. Plasma gastrin (A), RANKL (B), OPG/RANKL ratio (C), OPG (D), leptin (E), osteocalcin (F), and PTH (G) in H^+/K^+ATPase beta subunit knockout mice and controls. $^*P$-value < 0.05, $^{**}P$-value < 0.01 compared to age-matched controls.

CONCLUSION

H^+/K^+ATPase KO mice have gastric hypoacidity and have an osteoporotic phenotype at both 6 and 20 months age. The current results add to previous studies supporting a causal relationship between the use of PPI and the observed increase in fracture risk in patients. The mechanisms leading to osteoporosis are not established, but several studies suggest that reduced calcium absorption due to gastric hypoacidity could be most important.

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