Serum osteoprotegerin/osteoclastogenesis-inhibitory factor during pregnancy and lactation and the relationship with calcium-regulating hormones and bone turnover markers

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Abstract

Pregnancy and lactation induce dynamic changes in maternal bone and calcium metabolism. A novel cytokine termed osteoprotegerin (OPG)/osteoclastogenesis-inhibitory factor (OCIF) was recently isolated; this cytokine inhibits osteoclast maturation. To define the effects of pregnancy and lactation on circulating OPG/OCIF in mothers, we studied the changes in the levels of OPG/OCIF as well as those of calcium-regulating hormones and biochemical markers of bone turnover in the maternal circulation during pregnancy (at 8–11 weeks, at 22–30 weeks, at 35–36 weeks and immediately before delivery) and lactation (at 4 days and at 1 month postpartum).

Serum intact parathyroid hormone levels did not change and were almost within the normal range in this period. In contrast, serum 1,25-dihydroxyvitamin D levels increased with gestational age and were above the normal range during pregnancy. After delivery, they fell rapidly and significantly ($P<0.01$) to the normal range. The levels of serum bone-specific alkaline phosphatase, one of the markers of bone formation, increased with gestational age.

After delivery, these levels were further increased at 1 month postpartum. The levels at 1 month postpartum were significantly higher than those at 8–11 and 22–30 weeks of pregnancy ($P<0.01$ and $P<0.05$ respectively). The levels of serum C-terminal telopeptides of type I collagen, one of the markers of bone resorption, did not change during pregnancy. After delivery, they rapidly and significantly ($P<0.01$) rose at 4 days postpartum, and had then fallen by 1 month postpartum. Circulating OPG/OCIF levels gradually increased with gestational age and significantly ($P<0.01$) increased immediately before delivery to 1.40 ± 0.53 ng/ml (means $\pm$ S.D.) compared with those in the non-pregnant, non-lactating controls (0.58 ± 0.11 ng/ml). After delivery, they fell rapidly to 0.87 ± 0.27 ng/ml at 4 days postpartum and had fallen further by 1 month postpartum.

These results suggest that the fall in OPG/OCIF levels may be partially connected with the marked acceleration of bone resorption after delivery.

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Introduction

The structure and volume of bone is maintained by a continued and coordinated remodeling that involves bone resorption and subsequent bone formation. Estrogen deficiency in women, such as at menopause, is a major trigger of the changes in bone metabolism and causes the loss of bone density (Lufkin *et al.* 1992, Raize & Shoukri 1993). Some physiological states such as pregnancy or lactation also induce dynamic changes in bone and calcium metabolism in women. During pregnancy, especially in the third trimester, considerable amounts of calcium are transported from mother to fetus through the placenta for normal bone mineralization in the growing fetus. During lactation, calcium losses in mothers are greater than those during pregnancy. Nursing mothers provide an average of 200–250 mg calcium/day to their infants, sometimes as

much as 400 mg/day. In order to satisfy the increased demands for calcium, maternal metabolism of bone and calcium changes dynamically during pregnancy and lactation.

Recently, a novel cytokine termed osteoprotegerin (OPG)/osteoclastogenesis-inhibitory factor (OCIF) was purified from the conditioned medium of human embryonic lung fibroblasts IMR-90 (Tsuda *et al.* 1997). The administration of recombinant OPG/OCIF leads to an increase in bone mineral density, associated with a decrease in the number of active osteoclasts in normal rats (Yasuda *et al.* 1998), and it also prevents bone loss and restores bone strength in ovariectomized rats by reducing bone resorption (Simonet *et al.* 1997). It is further revealed that OPG/OCIF circulates in human blood mainly as a monomer, that serum concentrations of OPG/OCIF increase with age in both healthy men and women, and that

these concentrations are significantly higher in postmenopausal women with osteoporosis than in age-matched normal controls (Yano *et al.* 1999).

However, other physiological profiles or regulatory mechanisms of OPG/OCIF in women have not been clarified. To define the effects of pregnancy and lactation on circulating OPG/OCIF in mothers, we studied the changes in the levels of OPG/OCIF in the maternal circulation during pregnancy and after delivery. We further analyzed the relations between the circulating OPG/OCIF and calcium-regulating hormones or biochemical markers of bone turnover in mothers.

Materials and Methods

Experimental subjects

Fourteen Japanese women who attended the Tokushima University Hospital from an early stage of pregnancy, aged 23–36 years (mean $\pm$ S.D. 29.5 ± 4.4), with body mass indices (BMI) ranging from 17.1 to 27.9 kg/m² (mean $\pm$ S.D., 21.5 ± 3.8) were studied during pregnancy and after delivery after providing informed consent.

None of them had any disorders that affected their metabolism of calcium or bone, any history of endocrine, renal or liver illness, hypertension of pregnancy or gestational diabetes, and none was regularly taking medications or using hormonal contraceptives. All had had singleton pregnancies lasting 37 weeks or more and intended to breastfeed for at least 6 months.

Collection of blood samples

Fasting blood samples were collected during pregnancy (at 8–11 weeks, at 22–30 weeks, at 35–36 weeks of pregnancy and immediately before delivery, i.e. after the onset of labor pain) and after delivery (at 4 days and at 1 month postpartum). Serum was immediately separated after blood collection and promptly frozen at -40°C until assay.

Additional blood samples were collected from the follicular phase of 14 women who had a normal menstrual cycle, aged 21–39 years (mean $\pm$ S.D., 29.5 ± 5.0), with BMI ranging from 16.6 to 27.5 kg/m² (mean $\pm$ S.D., 21.5 ± 2.5), for the age- and BMI-matched controls of circulating OPG/OCIF after providing informed consent.

Determination of minerals, calcium-regulating hormones and bone turnover markers

Total calcium, albumin and phosphorus concentrations in sera were determined by an automatic analyzer (Olympus AU 2000 for calcium and albumin; Olympus AU 800 for phosphorus, Olympus Promarketing Inc., Tokyo, Japan). Before statistical analysis, all serum calcium values were adjusted for serum albumin concentration.

Serum intact parathyroid hormone (PTH) was measured by a two-site immunoradiometric assay (IRMA) (Nichols Research Institute, San Juan Capistrano, CA, USA), with a normal range of 10–65 pg/ml and an assay sensitivity of 1 pmol/l. Serum 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) was determined with a radioimmunoassay (RIA) kit (Immunodiagnostic Systems Ltd, Boldon, UK).

Markers of bone formation used in this study were osteocalcin and bone-specific alkaline phosphatase (BSAP). Serum osteocalcin was measured with an IRMA kit (Mitsubishi Chemical, Tokyo, Japan), with a normal range of 3.1–12.7 ng/ml and an assay sensitivity of 1 ng/ml. Serum BSAP was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Metra Biosystems Inc., Palo Alto, CA, USA). Average intra-assay variability was <5% for all measures of bone formation. A marker of bone resorption was C-terminal telopeptides of type I collagen (1 CTP) determined using an RIA kit (Orion Diagnostica, Espoo, Finland). Average intra-assay variability was <10%.

Measurement of circulating OPG/OCIF

Serum OPG/OCIF levels were determined using an ELISA kit (Cosmo Bio Co., Tokyo, Japan). Briefly, a serum sample was diluted ten times with specimen diluent supplied in this kit. The diluted specimen was pipetted into a reaction well coated with anti-human OPG/OCIF monoclonal antibody. During the incubation time, the anti-human OPG/OCIF monoclonal antibody immunologically binds the OPG/OCIF in the patient's specimen. After thorough washing, another anti-human OPG/OCIF monoclonal antibody conjugated with horseradish peroxidase was added to the reaction well and incubated with the coated antibody-antigen complex. The conjugated antibody binds to the OPG/OCIF antigen on the complex and makes an antibody-antigen-antibody complex by two steps (two-stepped sandwich method). After the second washing step, o-phenylenediamine solution containing hydrogen peroxide was added to the reaction well and, during an incubation period, a yellow color developed in proportion to the amount of enzyme conjugate bound to the well. The enzyme reaction was stopped by the addition of acid. The absorbance value of the specimens was determined using a spectrophotometer with the wavelength set at 492 nm. The assay range was 31.25–500 pg/ml. Average intra-assay variability was less than 10%.

Statistical analysis

Data are expressed as the means $\pm$ S.D. For the analyses of clinical and laboratory data, non-parametric analysis of variance (Kruskal–Wallis test) was used because of the presence of non-homogeneous variances across the group. The Scheffé's multiple range test was used for post hoc comparisons. Correlations between the serum

Figure 1 Mean $\pm$ S.D. changes in the levels of serum calcium and phosphorus during pregnancy and lactation in 14 women. The levels of serum calcium ($\circ$) did not change, while those of serum phosphorus ($\bullet$) at 4 days postpartum were significantly ($*P<0.05$) higher than those at 22–30 and 35–36 weeks of pregnancy.

concentrations of OPG/OCIF and calcium-regulating hormones or biochemical markers of bone metabolism were determined by linear regression analysis. All P values ≤ 0.05 were considered statistically significant. Analyses were carried out using a Stat Works program (Cricket Software, Inc., Philadelphia, PA, USA).

Results

Changes in minerals and calcium-regulating hormones

The changes in the levels of serum calcium and phosphorus during pregnancy and lactation are shown in Fig. 1. The levels of serum calcium did not change, while those of serum phosphorus at 4 days postpartum were significantly ($P<0.05$) higher than those at 22–30 and 35–36 weeks of pregnancy. The changes in the levels of circulating calcium-regulating hormones during pregnancy and lactation are shown in Fig. 2. The levels of serum intact PTH did not change statistically and were almost within the normal range. In contrast, the levels of serum $1,25(\text{OH})_2\text{D}$ increased with gestational age to 108.3 ± 20.9 pg/ml at 35–36 weeks and were above the normal range over the pregnancy. After delivery, they fell rapidly and significantly ($P<0.01$) at 4 days postpartum to the normal range.

Changes in markers of bone formation and resorption

The changes in the levels of the markers of bone formation and resorption during pregnancy and lactation are shown

Figure 2 Mean $\pm$ S.D. changes in the levels of circulating calcium-regulating hormones during pregnancy and lactation in 14 women. The standard levels are between the dotted lines. Serum intact parathyroid hormonal (PTH) levels did not change significantly and were almost within the normal range in this period. In contrast, the levels of serum $1,25(\text{OH})_2\text{D}$ increased with gestational age to 35–36 weeks and were above the normal range over the pregnancy. After delivery, they fell rapidly and significantly to the normal range. $*P<0.05$, $**P<0.01$ compared with 8–11 weeks.

in Fig. 3. Serum BSAP levels increased with gestational age. After delivery, these levels remained at the same level at 4 days postpartum, then further increased at 1 month postpartum. These levels at 1 month postpartum were significantly higher than those at 8–11 and 22–30 weeks of pregnancy ($P<0.01$ and $P<0.05$ respectively). Serum osteocalcin levels were low during pregnancy, and those in 12 of 14 samples at 22–30 weeks and those in nine immediately before delivery were below the sensitivity of the assay. They then continued to rise after delivery to 1 month postpartum. These levels at 1 month postpartum were significantly higher than those at all stages of pregnancy ($P<0.01$) and at 4 days postpartum ($P<0.05$).

Serum levels of 1 CTP did not change through pregnancy and were 5.0 ± 1.2 ng/ml immediately before delivery. After delivery, they rapidly and significantly ($P < 0.01$) rose to 28.9 ± 8.1 ng/ml at 4 days postpartum, then fell by 1 month postpartum to 8.5 ± 1.8 ng/ml.

Changes in OPG/OCIF

The levels of serum OPG/OCIF during pregnancy and lactation and in the controls are shown in Fig. 4. Serum

Figure 4 Mean $\pm$ s.d. changes in the levels of circulating osteoprotegerin/osteoclastogenesis-inhibitory factor (OPG/OCIF) during pregnancy and lactation in 14 women and in the controls (14 non-pregnant, non-lactating women). Serum OPG/OCIF levels steadily increased with gestational age during pregnancy. The levels immediately before delivery were significantly ($**P < 0.01$) higher than those in the controls. After delivery, they fell rapidly at 4 days postpartum and decreased furthermore to 1 month postpartum.

OPG/OCIF levels in the controls were 0.58 ± 0.11 ng/ml. They steadily increased with gestational age during pregnancy. These levels immediately before delivery were 1.40 ± 0.53 ng/ml and were significantly ($P < 0.01$) higher than those in the controls. After delivery, they fell rapidly to 0.87 ± 0.27 ng/ml at 4 days postpartum and decreased furthermore to 1 month postpartum.

Relationship between circulating OPG/OCIF and bone turnover markers or calcium-regulating hormones

OPG/OCIF values in the maternal circulation did not correlate with any single parameter of the bone turnover

Figure 3 Mean $\pm$ s.d. changes in the levels of the markers of bone formation and resorption during pregnancy and lactation in 14 women. The standard levels are between the dotted lines for bone-specific alkaline phosphatase (BSAP) and osteocalcin, and under the dotted line for C-terminal telopeptides of type 1 collagen (1 CTP). Serum BSAP levels increased with gestational age. After delivery, these remained at the same level at 4 days postpartum, then further increased at 1 month postpartum. The levels at 1 month postpartum were significantly higher than those at 8–11 and 22–30 weeks of pregnancy ($P < 0.01$ and $P < 0.05$ respectively). Serum osteocalcin levels were low during pregnancy, and those in 12 of 14 samples at 22–30 weeks and those in nine immediately before delivery were below the sensitivity of the assay. The values below the sensitivity of the assay were estimated as 1 ng/ml. After delivery they continued to rise to 1 month postpartum. Serum levels of 1 CTP did not change through pregnancy. After delivery, they rapidly and significantly rose at 4 days postpartum, then fell by 1 month postpartum. $*P < 0.05$, $**P < 0.01$ compared with 8–11 weeks.

markers or calcium-regulating hormones (data not shown).

Discussion

Osteoporosis is a metabolic bone disease and it is associated with the imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. Postmenopausal estrogen deficiency and aging are the two main factors which cause osteoporosis in women. In addition, particular physiological states such as pregnancy or lactation induce dynamic changes in maternal calcium metabolism. During these physiological states, the demands for calcium are large because of the growing fetal and infantile skeletons. The loss of calcium from a mother to a fetus through the placenta is about 30 g over pregnancy (Pitkin 1985). In contrast, the loss of calcium from a mother to an infant is about 40 g during 6 months of lactation. The loss of bone density in the spine and hip averages 4–6% during the first 6 months of lactation (Hayslip *et al.* 1989, Affinito *et al.* 1996, Lopez *et al.* 1996, Kalkwarf *et al.* 1997). Calcium is mobilized from the maternal skeleton to maintain serum calcium concentrations within a narrow range and to support milk production. Although the demands for calcium in the late stages of pregnancy and lactation are similar at approximately 300 mg/day (Laskey *et al.* 1998), the loss of bone density is none or little during pregnancy (Kent *et al.* 1990, 1991) while it is significant in lactation (Atkinson & West 1970, Chan *et al.* 1982). One probable reason why pregnancy does not affect maternal bone density is the increased capacity of intestinal calcium absorption due to the increased circulating $1,25(\text{OH})_2\text{D}$ (Kent *et al.* 1990, 1991). Another reason may be the protective effects of increased circulating estrogen on the bone. However, other details of the mechanisms are still not known.

A novel cytokine OCIF, a secreted protein consisting of 380 amino acids, was isolated as a basic glycoprotein with apparent molecular weights of 60 kDa for a monomer and 120 kDa for the homodimer (Tsuda *et al.* 1997) and is also called OPG. cDNA encoding OCIF has been cloned, and analysis of the cDNA sequence revealed that OCIF is a soluble member of the tumor necrosis factor (TNF) receptor family (Yasuda *et al.* 1998). OPG/OCIF competes with the receptor activator of NF- κ B for binding to osteoclast differentiation factor, and it inhibits osteoclast maturation *in vivo* and *in vitro*. Bekker *et al.* (2001) showed that a single s.c. injection of OPG leads to a rapid decrease in urinary N-telopeptide and to a delayed decrease in BSAP (Yano *et al.* 1999). Yano *et al.* (1999) reported that OPG/OCIF circulates in human blood mainly as a monomer (Bekker *et al.* 2001). Because the ELISA kit for the determination of OPG/OCIF used in this study can measure monomeric and dimeric OPGs/OCIFs equally, it is a suitable assay for the determination of serum concentrations of OPG/OCIF. It has also been reported that

serum concentrations of OPG/OCIF increase with age in both healthy men and women, and that these concentrations are significantly higher in postmenopausal women with osteoporosis than in age-matched normal controls (Yano *et al.* 1999). Browner *et al.* (2001) reported that serum OPG levels in elderly women are associated with diabetes and with cardiovascular mortality, but not with baseline bone mineral density or with subsequent strokes or fractures. It was also reported that in men more than 40 years of age serum concentrations of OPG were negatively correlated with urinary excretion of total deoxypyridinoline (Dpd), but not with biochemical markers of bone formation (Szulc *et al.* 2001). Other details of the profiles of serum OPG/OCIF in women are still not known. We therefore examined the changes in the serum concentrations of OPG/OCIF during pregnancy and lactation.

Our results revealed that the concentrations of serum OPG/OCIF in mothers steadily increased during pregnancy and the levels immediately before delivery were about 2.5 times as high as those in non-pregnant, non-puerperal women. Then they fell rapidly and significantly after delivery. It has been reported that the concentration of circulating OPG/OCIF in the mouse markedly increases during pregnancy (Yano *et al.* 2001). From these results, it can be suggested that circulating OPG/OCIF may play an important role in bone metabolism during pregnancy in mammals. Simonet *et al.* (1997) reported that OPG/OCIF mRNA expression in the placenta is strong in the mouse and the human, and suggested that the sequential expression of the OPG/OCIF gene in maternal tissues such as decidua and placenta may play a role in the control of bone metabolism in a pregnant female. The main reason that circulating OPG/OCIF levels were high during pregnancy from this point is thought to be production by the placenta. The levels of circulating OPG/OCIF rose sharply from 35–36 weeks to immediately before delivery. This might be a result of the larger loss of calcium in mothers in the late stages of pregnancy.

Our study also showed that serum levels of BSAP, one of the markers of bone formation, increased during pregnancy and were above the normal range during pregnancy. After delivery, these levels remained at the same level at 4 days postpartum, then further increased at 1 month postpartum. It is widely recognized that osteocalcin is a sensitive and specific clinical marker of bone turnover in most situations (Price *et al.* 1980, Gundberg *et al.* 1983). However, serum osteocalcin measurements are not useful as a marker of bone turnover during pregnancy because serum osteocalcin may be destroyed by a placental mechanism (Rodin *et al.* 1989). In this study, serum osteocalcin levels were low during pregnancy, and especially those in 12 of 14 samples at 22–30 weeks and those in nine immediately before delivery which were below the sensitivity of the assay. These results are consistent with previous reports (Martinez *et al.* 1985, Cole *et al.* 1987, Rico *et al.* 1987, Rodin *et al.* 1989). Serum levels of 1

CTP, one of the markers of bone resorption, did not change through pregnancy. These levels remained within the standard level until 35–36 weeks of pregnancy and were slightly over the standard level immediately before delivery. After delivery, they rapidly and markedly rose at 4 days postpartum. Naylor *et al.* (2000) reported that the levels of urinary pyridinoline (Pyd) and Dpd, which are the urinary markers of bone resorption, were higher during pregnancy than those in the non-pregnant, non-lactating period and increased with gestational age. After delivery, these levels further increased (Naylor *et al.* 2000). During pregnancy, the changes in the levels of serum 1 CTP were quite different from those of urinary Pyd and Dpd. Serum 1 CTP may be decomposed to some degree by a placental mechanism the same as serum osteocalcin. In our results and those of Naylor *et al.* (2000), bone formation and resorption were accelerated during pregnancy, especially in the late stages. After delivery, bone resorption markedly rose at 4 days postpartum and fell at 1 month postpartum, while bone formation remained increased at 4 days postpartum and was still increased at 1 month postpartum. It has been reported that calcium supplementation cannot prevent the loss of bone density and does not affect calcium homeostasis and bone turnover in lactating women (Kalkwarf *et al.* 1997, 1999). Therefore, loss of calcium induced by lactation may not be the only cause of the loss of bone density in lactating women. Lactating women show lower estrogen levels, higher prolactin levels and higher PTH-related peptide levels than non-lactating women (Sowers *et al.* 1996). These conditions must be the cause of the loss of bone density during lactation. Other triggers by which bone resorption accelerates after delivery are the decrease of $1,25(\text{OH})_2\text{D}$ and may be the fall of OPG/OCIF, according to our results.

In our analyses of cord blood profiles, the levels of calcium, phosphorus and the biomarkers of both bone formation and resorption were quite high, while those of intact PTH were low and those of $1,25(\text{OH})_2\text{D}$ were within the normal range. Mean OPG/OCIF levels in cord blood were 0.35 ng/ml and lower than those in adult women (data not shown). Low levels of OPG/OCIF may be one of the factors of high bone turnover in fetuses.

In summary, we have revealed changes in the levels of OPG/OCIF as well as in those of calcium-regulating hormones and biochemical markers of bone turnover in maternal circulation during pregnancy and lactation. We found that the fall in OPG/OCIF levels may be partially connected with the marked acceleration of bone resorption after delivery. Further studies are necessary to elucidate the detailed functions of OPG/OCIF on bone metabolism during pregnancy and lactation.

References

- Affinito P, Tommaselli GA, di Carlo C, Guida F & Nappi C 1996 Changes in bone mineral density and calcium metabolism in breastfeeding women: a one year follow-up study. *Journal of Clinical Endocrinology and Metabolism* **81** 2314–2318.
- Atkinson PJ & West RR 1970 Loss of skeletal calcium in lactating women. *Journal of Obstetrics and Gynaecology of the British Commonwealth* **77** 555–560.
- Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT & Dunstan CR 2001 The effect of a single dose of osteoprotegerin in postmenopausal women. *Journal of Bone and Mineral Research* **16** 348–360.
- Browner WS, Lui LY & Cummings SR 2001 Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. *Journal of Clinical Endocrinology and Metabolism* **86** 631–637.
- Chan GM, Ronald N, Slater P, Hollis J & Thomas MR 1982 Decreased bone mineral status in lactating adolescent mothers. *Journal of Pediatrics* **101** 767–770.
- Cole DE, Gundberg CM, Stirk LJ, Atkinson SA, Hanley DA, Ayer LM & Baldwin LS 1987 Changing osteocalcin concentrations during pregnancy and lactation: implications for maternal mineral metabolism. *Journal of Clinical Endocrinology and Metabolism* **65** 290–294.
- Gundberg CM, Lian JB, Gallop PM & Steinberg JJ 1983 Urinary gamma-carboxyglutamic acid and serum osteocalcin as bone markers: studies in osteoporosis and Paget's disease. *Journal of Clinical Endocrinology and Metabolism* **57** 1221–1225.
- Hayslip CC, Klein TA, Wray HL & Duncan WE 1989 The effects of lactation on bone mineral content in healthy postpartum women. *Obstetrics and Gynecology* **73** 588–592.
- Kalkwarf HJ, Specker BL, Bianchi DC, Ranz J & Ho M 1997 The effect of calcium supplementation on bone density during lactation and after weaning. *New England Journal of Medicine* **337** 523–528.
- Kalkwarf HJ, Specker BL & Ho M 1999 Effects of calcium supplementation on calcium homeostasis and bone turnover in lactating women. *Journal of Clinical Endocrinology and Metabolism* **84** 464–470.
- Kent GN, Price RI, Gutteridge DH, Smith M, Allen JR, Bhagat CI, Barnes MP, Hickling CJ, Retallack RW, Wilson SG, Devlin RD, Davies C & St John A 1990 Human lactation: forearm trabecular bone loss, increased bone turnover, and renal conservation of calcium and inorganic phosphate with recovery of bone mass following weaning. *Journal of Bone and Mineral Research* **5** 361–369.
- Kent GN, Price RI, Gutteridge DH, Rosman KJ, Smith M, Allen JR, Hickling CJ & Blakeman SL 1991 The efficiency of intestinal calcium absorption is increased in late pregnancy but not in established lactation. *Calcified Tissue International* **48** 293–295.
- Laskey MA, Prentice A, Hanratty LA, Jarjou LM, Dibba B, Beavan SR & Cole TJ 1998 Bone changes after 3 mo of lactation: influence of calcium intake, breast-milk output, and vitamin D-receptor genotype. *American Journal of Clinical Nutrition* **67** 685–692.
- Lopez JM, Gonzalez G, Reyes V, Campino C & Diaz S 1996 Bone turnover and density in healthy women during breastfeeding and after weaning. *Osteoporosis International* **6** 153–159.
- Lufkin EG, Wahner HW, O'Fallon WM, Hodgson SF, Kotowicz MA, Lane AW, Judd HL, Caplan RH & Riggs BL 1992 Treatment of postmenopausal osteoporosis with transdermal estrogen. *Annals of Internal Medicine* **117** 1–9.
- Martinez ME, de Pedro C, Catalan P, Salinas M, Balaguer G & Ordas J 1985 Levels of osteocalcin in normal pregnancy. *American Journal of Obstetrics and Gynecology* **153** 708–709.
- Naylor KE, Iqbal P, Fledelius C, Fraser RB & Eastell R 2000 The effect of pregnancy on bone density and bone turnover. *Journal of Bone and Mineral Research* **15** 129–137.
- Pitkin RM 1985 Calcium metabolism in pregnancy and the perinatal period: a review. *American Journal of Obstetrics and Gynecology* **151** 99–109.

- Price PA, Parthemore JG & Deftos LJ 1980 New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. *Journal of Clinical Investigation* **66** 878–883.
- Raize LG & Shoukri KC 1993 Pathogenesis of osteoporosis. In *Physiology and Pharmacology of Bone*, pp 299–323. Eds GR Mundy & TJ Martin. Heidelberg: Springer-Verlag.
- Rico H, Cabrane JA, Farinas M, Costalles C, Martin F & Gaaebelt K 1987 Serum osteocalcin at delivery. *Journal of Obstetrics and Gynecology* **7** 259–261.
- Rodin A, Duncan A, Quartero HW, Pistofidis G, Mashiter G, Whitaker K, Crook D, Stevenson JC, Chapman MG & Fogelman I 1989 Serum concentrations of alkaline phosphatase isoenzymes and osteocalcin in normal pregnancy. *Journal of Clinical Endocrinology and Metabolism* **68** 1123–1127.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P & Boyle WJ 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89** 309–319.
- Sowers MF, Hollis BW, Shapiro B, Randolph J, Janney CA, Zhang D, Schork A, Crutchfield M, Stanczyk F & Russell-Aulet M 1996 Elevated parathyroid hormone-related peptide associated with lactation and bone density loss. *Journal of the American Medical Association* **276** 549–554.
- Szulc P, Hofbauer LC, Heufelder AE, Roth S & Delmas PD 2001 Osteoprotegerin serum levels in men: correlation with age, estrogen, and testosterone status. *Journal of Clinical Endocrinology and Metabolism* **86** 3162–3165.
- Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T & Higashio K 1997 Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochemical and Biophysical Research Communications* **234** 137–142.
- Yano K, Tsuda E, Washida N, Kobayashi F, Goto M, Harada A, Ikeda K, Higashio K & Yamada Y 1999 Immunological characterization of circulating osteoprotegerin/osteoclastogenesis inhibitory factor: increased serum concentrations in postmenopausal women with osteoporosis. *Journal of Bone and Mineral Research* **14** 518–527.
- Yano K, Shibata O, Mizuno A, Kobayashi F, Higashio K, Morinaga T & Tsuda E 2001 Immunological study on circulating murine osteoprotegerin/osteoclastogenesis inhibitory factor (OPG/OCIF): possible role of OPG/OCIF in the prevention of osteoporosis in pregnancy. *Biochemical and Biophysical Research Communications* **288** 217–224.
- Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T & Higashio K 1998 Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. *Endocrinology* **139** 1329–1337.

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