DORIS HOLMBERG-MARTTILA

Maternity and Bone

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ACADEMIC DISSERTATION
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To Tommi,
Johannes, Helena and Johanna

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to as I-V in the text. In addition, some previously unpublished information is also presented.

I   Holmberg-Marttila D, Sievänen H and Tuimala R (1999): Changes in bone mineral density during pregnancy and postpartum: prospective data of five women. Osteoporosis Int 10:41-46.

II   Holmberg-Marttila D and Sievänen H (1999): Prevalence of bone mineral changes during postpartum amenorrhea and after resumption of menstruation. Am J Obstet Gynecol 180:537-538.

III   Holmberg-Marttila D, Sievänen H, Järvinen TL and Järvinen TA (2000): Vitamin D and estrogen receptor polymorphisms and bone mineral changes in postpartum women. Calcif Tissue Int 66:184-189.

IV   Holmberg-Marttila D, Sievänen H, Laippala P and Tuimala R (2000): Factors underlying changes in bone mineral during postpartum amenorrhea and lactation. Osteoporosis Int 11:570-576.

V   Holmberg-Marttila D, Leino A and Sievänen H: Bone turnover markers in relation to lactation, postpartum amenorrhea and resumption of menstruation. Submitted for publication.

ABBREVIATIONS

Alb	Albumin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BAP	Bone-specific alkaline phosphatase
BF	Breast-feeding
BMD	Areal bone mineral density, g/cm^2
BMI	Body mass index, kg/m^2
Ca	Calcium
CI	Confidence interval
CTx	Type I collagen carboxy-terminal telopeptide
CV	Coefficient of variation
DPA	Dual photon absorptiometry
DXA	Dual energy X-ray absorptiometry
E_2	Estradiol
ER	Estrogen receptor
FF	Formula-feeding
FSH	Follicle-stimulating hormone
LH	Luteinising hormone
OC	Osteocalcin
PPA	Postpartum amenorrhea
Pi	Inorganic phosphate
PINP	Amino-terminal propeptide of type I procollagen
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
QCT	Quantitative computed tomography
RDA	Recommended dietary allowance
SD	Standard deviation
SEM	Standard error of mean

SPA	Single photon absorptiometry
T_R	Estimated time of recovery to postpregnancy level
TRAP	Tartrate-resistant acid phosphatase
VDR	Vitamin D receptor
25OHD	25 hydroxyvitamin D
$1,25(OH)_2D$	1,25 dihydroxyvitamin D vitamin

INTRODUCTION

Women who breastfeed have chosen their feeding preference after considering both infant and maternal needs. For infants, breast milk provides nutritional (Prentice 1991, Lawrence 1994) and cognitive (Lucas et al. 1992, Rogan and Gladen 1993) benefit, and enhanced immunologic protection against many infectious diseases (Howie et al. 1990, Bock and Sampson 1994). On one hand, breastfeeding is a cost-effective means of feeding for mothers, a convenience in that the milk requires no separate preparation and handling, and a unique opportunity for bonding with their offspring (Kennedy 1994). On the other, however, a potential reduction in bone mass associated with lactation has recently received considerable attention (Sowers 1996a, 1996b, Tudor-Locke and McColl 2000).

Low bone mass is a major determinant of osteoporotic fractures (Riggs and Melton 1992, Dempster and Lindsay 1993, Johnell 1996). Osteoporosis, one of the major public health problems world-wide (Riggs and Melton 1995), is a bone disease characterised by low bone mass and microarchitectural deterioration of bone tissue which leads to increased bone fragility and a consequent increase in fracture risk (Consensus Conference on Osteoporosis 1993). The risk of evolving skeletal fragility is dependent on the maximum amount of bone mass achieved during childhood and adolescence (peak bone mass) and the amount and rate of bone loss thereafter. A high peak bone mass can be thus seen as a safety margin to maintain adequate bone strength in the face of inevitable menopausal and age-related bone losses (Law et al. 1991, Johnston and Slemenda 1992, Eisman et al. 1993).

Existing knowledge of premenopausal bone mass is inadequate to demonstrate precisely the natural course of bone mass evolution and variance in it prior to menopause. The accelerated bone loss in women is most distinct during the first decade after the menopause, involving a disproportionate loss of cancellous bone, and is mediated mainly by the depletion in the direct restraining effects of estrogen on bone cell function (Slemenda et al. 1987, Riggs et al. 1998). Needless to say, estrogen exposure is also one of the most important determinants of bone mass in premenopausal females (Armamento-Villareal et al. 1992, Slemenda et al.

1996). Prolonged periods of amenorrhea can be regarded as a risk factor for the evolution of osteoporosis, and in the majority of amenorrheic premenopausal women, estrogen deficiency has been hypothesised to be the factor through which amenorrhea affects bone mass (Schachter and Shoham 1994, Miller and Klibanski 1999).

Pregnancy and lactation are physiological phases of life characterised by significant alterations in maternal endocrine function, particularly regarding estrogen and prolactin levels (McNeilly 1993, Sowers 1996b). Moreover, pregnancy and lactation pertain to circumstances in which the mother must provide for maintenance of her own skeleton as well as for the construction of her child. Specifically, during the 9 months of pregnancy she provides the fetus with 25-30 g of calcium, and during the ensuing 9 months or so of lactation, she provides another 50-75 g in breast milk (Heaney 1996). Evidence of the effect of pregnancy on bone mineral density (BMD) is as yet somewhat inconclusive and scanty (Naylor et al. 2000, Drinkwater and Chesnut 1991, Sowers et al. 1991b, Kent et al. 1993, Cross et al. 1995a, Ritchie et al. 1998). In contrast, recent prospective studies have mainly reported 4% to 7% decreases in lumbar BMD and 3% to 5% decreases in femoral neck BMD during 6 months of lactation (Sowers et al. 1993, Kalkwarf and Specker 1995, Lopez et al. 1996, Laskey and Prentice 1997), and either partial (Sowers et al. 1993, Affinito et al. 1996) or complete (Lopez et al. 1996, Krebs et al. 1997, Caird et al. 1994, Kalkwarf and Specker 1995, Laskey and Prentice 1997) recovery after weaning. The complete recovery seems to be more evident at lumbar spine than at proximal femur (Sowers et al. 1993, Kolthoff et al. 1998, Laskey and Prentice 1999). As to whether this bone loss, if it occurs, is permanent the existing retrospective data provide no conclusive evidence (Hreshchyshyn et al. 1988, Lissner et al. 1991, Feldblum et al. 1992, Melton et al. 1993). Little is likewise known regarding the specific mechanism underlying bone turnover and skeletal changes during human reproduction.

Many questions concerning the factors affecting bone loss and recovery during reproduction have remained unanswered. Specifically, the role of postpartum amenorrhea (PPA) is unclear. Only a few studies have primarily considered resumed menstruation as a potential

regulator of bone metabolism during the postpartum period (Caird et al. 1994, Kalkwarf and Specker 1995, Sowers et al. 1996, Kolthoff et al. 1998). These studies have indicated that lactating women whose menstruation is resumed earlier suffer lesser bone loss and make a better recovery after weaning than lactating women with a longer period of amenorrhea. This issue is complex to interpret because women who resume menstruation early tend also to lactate for shorter periods of time. To introduce a new viewpoint in postpartum studies, we hypothesised that BMD changes are driven by hypoestrogenemia during amenorrhea. BMD was therefore assessed on a physiology-based schedule with the main emphasis on PPA, since the normal ovulatory ovarian function in postpartum mothers is reported to be suppressed until the resumption of menstruation (Howie et al. 1982, Diaz et al. 1992). The goal of the present study was to examine changes in BMD and bone turnover during PPA and the subsequent follow-up period after resumed menstruation, and to expose genetic, physiological and habitual factors which would best account for these changes.

REVIEW OF THE LITERATURE

1. The course of premenopausal bone mass

In adults, bone mass present at any time in life is a function of the amount achieved by maturity and that lost with ageing and concomitant events. The traditional view has been that the peak bone mass is attained as late as the third decade of life (Recker et al. 1992). However, recent prospective data indicate that most of the bone mass in multiple skeletal regions is already accumulated by late adolescence, that is by about the age of twenty (Katzman et al. 1991, Theintz et al. 1992, Kröger et al. 1993). There is more controversy regarding the maintenance of bone mass during the adult premenopausal years (Baran 1994). Peak bone mass in the lumbar spine, femoral neck and distal radius is held either to remain stable until the menopause (Mazess and Barden 1991, Slosman et al. 1994, Slemenda et al. 1996, Sowers et al. 1998a, Chapurlat et al. 2000), or to start to decrease during the fourth decade of life at a rate of 1.0% per premenopausal year (Smith et al. 1989, Baran et al. 1989, Sowers et al. 1992b, Luckey et al. 1996, Prior et al. 1996, Melton et al. 2000). Recent studies also suggest that premenopausal bone loss occurs earlier in the femoral neck than in the lumbar spine (Sowers et al. 1998a, Hui et al. 1999, Melton et al. 2000).

2. Bone mass determinants

2.1. Genetics

Genetic factors have been shown to exert a strong influence on BMD accounting for as much as 80% of the age-specific variation (Pocock et al. 1987, Seeman et al. 1989, Slemenda et al. 1991). Heritability affects not only the attainment of peak bone mass (Lutz and Tesar 1990, Sowers et al. 1992a, Hansen et al. 1992, Seeman et al. 1994) but also the subsequent rate of bone loss (Lutz and Tesar 1990, Kelly et al. 1993) and bone turnover

(Kelly et al. 1991, Hansen et al. 1992). However, the contribution of heritability seems to depend on particular bone sites, showing a weaker influence in the appendicular skeleton than in central sites (Pocock et al. 1987, Seeman et al. 1989, Kelly et al. 1993, Krall and Dawson-Hughes 1993). Although genetic factors are currently thought to make a major contribution to the total inter-individual variance observed in BMD, gene-environment interaction should not be ignored (Slemenda et al. 1991, Kelly et al. 1993, Salamone et al. 1996b).

The variation in population-based BMD suggests polygenic inheritance (Eisman 1995, Peacock 1995). Among the candidate genes involved in the regulation of bone mass, greatest attention has focused on the vitamin D receptor (VDR) gene. The polymorphism of the VDR gene was originally held to account for up to 75% of the genetic effect (Morrison et al. 1994), this manifesting itself as a considerably lower BMD in the BB homozygote (without the restriction enzyme *BsmI* sites on the two VDR gene alleles) than in the bb genotype. More recently the VDR theory has been under a matter of lively debate (Eisman 1995, Peacock 1995, Cooper and Umbach 1996). The majority of studies on premenopausal women, however, indicate that the VDR polymorphism is indeed to some extent associated with bone mass (Barger-Lux et al. 1995, Fleet et al. 1995, Salamone et al. 1996a, Tokita et al. 1996, Viitanen et al. 1996), but this finding is not consistent (Garnero et al. 1995, Willing et al. 1998). Neither would there seem to be conclusive evidence for a relationship between VDR genotype and premenopausal bone turnover (Fleet et al. 1995, Howard et al. 1995, Garnero et al. 1995, Tokita et al. 1996), or the rate of premenopausal bone changes, which have been reported either to relate (Yamagata et al. 1994) or not to relate (Järvinen et al. 1998, Willing et al. 1998) to allelic variation in VDR.

The estrogen receptor (ER) gene polymorphism is another potential candidate for genetic regulation of BMD. Kobayashi and colleagues (1996) demonstrated that the restriction fragment length polymorphisms at the first intron of the ER gene (defined by the *XbaI* or *PvuII* restriction enzymes) were associated with variation in BMD in postmenopausal women. Results of the few subsequent studies on this relationship in premenopausal women

have been contradictory (Mizunuma et al. 1997, Ongphiphadhanakul et al. 1998, Willing et al. 1998, Han et al. 1999). Two of them (Mizunuma et al. 1997, Willing et al. 1998) also paid attention to the rate of bone change, finding no significant genotypic differences in BMD changes. It is highly likely that the polymorphisms of several important genes affecting bone metabolism (for example VDR and ER) could have interactive effects, the relationship between the variability in BMD and genetic factors thus possibly being even more complicated than previously envisaged. Not surprisingly, a significant additive effect of VDR and ER polymorphisms on BMD has been identified (Willing et al. 1998).

2.2. Body weight

BMD, by definition, is strongly associated with bone size (Sievänen 2000) and therefore also with body size and body weight (Sowers et al. 1991a, Mazess and Barden 1991, Reid et al. 1992, Lindsay et al. 1992). Thus, should the most important influence on bone mass arise from the load the bone had to bear (Frost 1993, Lanyon 1996), it is possible that a substantial reduction in body weight could initiate changes in bone remodelling which might eventually lead to a detectable decrease in BMD. In premenopausal overweight women a significant loss in body mass has been accompanied by significant losses in total body BMD (Compston et al. 1992, Jensen et al. 1994, Ramsdale and Bassey 1994, Van Loan et al. 1998, Fogelholm et al. in press), whereas some studies have found no significant changes in total body BMD (Hendel et al. 1996, Andersen et al. 1997). Data on specific bone sites suggest that BMD declines more rapidly in predominantly trabecular bones than in more cortical bones (Ramsdale and Bassey 1994), albeit, not in a consistent fashion (Andersen et al. 1997). Little is known of the effect of changes in body weight on BMD in normal-weight populations. Non-obese premenopausal women have shown higher rates of BMD loss in the lumbar spine and hip even with a modest weight loss (Salamone et al. 1999). Nor is it clear whether BMD recovers with weight regain after weight reduction, and if so, to what extent (Compston et al. 1992, Jensen et al. 1994, Fogelholm et al. in press). It is important to note that longitudinal effects of body weight are complicated to interpret in that changes in BMD observed with weight loss may be true, or may result partly or totally from the

methodological inability of dual- energy X-ray absorptiometry (DXA) to deal properly with body composition changes (Bolotin 1998, Van Loan et al. 1998, Bolotin et al. in press).

2.3. Nutrition

Nutritional factors are essential for the maintenance of bone mass, these including dietary intake of energy, protein, calcium, phosphorus, vitamin D and many other nutrients (reviewed by Anderson 1992, Sowers and Galuska 1993, Kruger and Horrobin 1997, Tudor-Locke and McColl 2000). In adults in industrialised nations the most important nutrients for bone health are calcium and vitamin D (Heaney 1996).

There is little epidemiologic evidence on nutrients other than calcium in premenopausal women (Tudor-Locke and McColl 2000). Calcium is vital for the skeleton, but there is controversy as to how much calcium is required. Numerous studies have examined the relationship between calcium, nutrition and bone mass. The inconsistencies in the literature have been extensively reviewed (Cumming 1990, Heaney 1992, Baran 1994, Welten et al. 1995, Tudor-Locke and McColl 2000). In the case of premenopausal women there are only a few controlled intervention studies of calcium supplementation showing a protective effect against bone loss in the lumbar spine (Baran et al. 1989) and the humerus (Smith et al. 1989). Although many longitudinal studies have failed to show any association of calcium intake with premenopausal bone mass and/ or BMD changes (Riggs et al. 1987, Mazess and Barden 1991, Sowers et al. 1992b, Citron et al. 1995), the evidence from the meta-analyses undertaken by Cumming (1990) and Welten and colleagues (1995) indicates that calcium intake is positively associated with bone mass in premenopausal women. However, it has been proposed that calcium functions as a threshold nutrient (Matkovic and Heaney 1992, Heaney 1996): i.e. calcium intake is relevant up to a certain threshold intake only, which varies between 1000 mg/day and 1500 mg/day depending on age, and higher calcium allowances seem to have only a minor, if any additional effect on bone.

Vitamin D plays an important role in the regulation of calcium and phosphorus absorption and skeletal mineralisation (Nordin and Morris 1992). If levels of vitamin D are insufficient, parathyroid hormone (PTH) secretion is increased, which leads to increased osteoclastic activation frequency. Vitamin D deficiency causes rickets in children and osteomalacia in adults. An adequate source of vitamin D of at least 400 IU per day contributes to bone health (Holick 1996). The relative contributions of orally ingested and dermally synthesised vitamin D are poorly characterised, and the latter is very difficult to measure (Heaney 2000). However, it is estimated that upwards 80% to 90% of the body's requirement for vitamin D comes from exposure to sunlight (Holick 1994), and incidental sun exposure during the summer months normally ensures adequate vitamin D synthesis in premenopausal women (Holick 1996, Tudor-Locke and Mc Coll 2000).

2.4. Life style

The importance of physical activity and exercise and the deleterious effects of immobilisation on bone and in the maintenance of skeletal mass are well established and have been reviewed by several authors (Schoutens et al. 1989, Forwood and Burr 1993, Chilibeck et al. 1995, Snow et al. 1996, Järvinen and Kannus 1997, Tudor-Locke and McColl 2000). Overall, physically active subjects have, according to these reviews, significantly higher BMD than age-matched sedentary controls. Most striking evidence for a positive effect of physical activity on bone arises from cross-sectional comparisons between athletes and their non-athlete counterparts, showing that athletes may have substantially greater BMD than non-athletes (e.g. Heinonen et al. 1996). There are also sufficient longitudinal data to demonstrate that moderate to intensive training programs have a positive effect on BMD (see also the meta-analyses by Wolff et al. 1999, Wallace and Cumming 2000). The effects of exercise are very likely load-dependent and site-specific (Kannus et al. 1996). The role of non-athletic exercise in maintaining or improving BMD has remained more inconclusive. Low or moderate additional exercise in young women may be of no value in further increasing bone mass if they are already physically active (Sinaki et al. 1996). Out of a few prospective studies investigating the relationship between current or

lifetime physical activity and BMD in premenopausal adults, one has reported a positive association (Recker et al. 1992), whereas some have found no association (Mazess and Barden 1991, Sowers et al. 1992b). It is of note, that the effect of physical activity may be evident only with adequate calcium intakes (Specker 1996).

High caffeine consumption has been proposed as a risk factor for osteoporotic fracture, but the evidence associating high caffeine intake with low BMD is inconsistent (reviewed by Laitinen and Välimäki 1993, Tudor-Locke and McColl 2000). In premenopausal women no significant relationship between caffeine intake and bone mass has been observed (Packard and Recker 1996). The potential bone benefits of tea consumption warrant also further investigation (Yang and Landay 2000).

Alcohol would appear to have direct toxic effects on bone and mineral metabolism, and alcohol abuse is known to be associated with deleterious changes in bone structure and a decrease in BMD (reviewed by Laitinen and Välimäki 1993, Tudor-Locke and McColl 2000). In contrast, more moderate alcohol intake is unlikely to be associated with lower BMD in premenopausal women (Sowers et al. 1992b). Even a positive correlation of moderate to heavy alcohol consumption with bone density in premenopausal women has been reported (Holbrook and Barrett-Connor 1993).

No longitudinal study has assessed the effects of smoking on bone in premenopausal women. The estimate from a recent meta-analysis of the influence of cigarette smoking on BMD indicated no deficit in premenopausal bone mass, but the effect of smoking on BMD seems to increase cumulatively with age (Law and Hackshaw 1997), thus resulting in increased bone loss after menopause and hip fractures in older women. Interestingly, current smoking may be associated with substantial deficits in bone mass in women with a body mass index (BMI) <25 kg/m^2 or a with breast-feeding history (Jones and Scott 1999). The data on effects of smoking, caffeine and alcohol on BMD are difficult to evaluate reliably and are not very convincing (reviewed by Sowers and Galuska 1993).

2.5 Hormonal factors

Regulation of bone mineral homeostasis pertains to control of the intra- and extracellular levels of calcium, magnesium and phosphate ions with calciotropic hormones, i.e., parathyroid, calcitonin and D-vitamin hormones acting mainly on three target tissues, i.e. bone, intestine and kidney (Bikle 1993). Osteoblasts have receptors for parathyroid hormone (PTH) and 1,25 dihydroxyvitamin D vitamin ($1,25(OH)_2D$), and osteoclasts for calcitonin (Einhorn 1996). Other systemic hormones regulating bone remodelling include growth hormone, insulin, thyroid hormones, glucocorticoids and sex steroids (Canalis 1993). Estrogen receptors, for example, have been found in all main types of bone cells (Eriksen et al. 1988, Komm et al. 1988, Pensler et al. 1990, Oursler et al. 1991).

Of the sex steroids, progesterone (Prior et al. 1990) and androgens (Slemenda et al. 1996) may affect BMD, but estrogen obviously plays the vital role in the growth and maturation of bone as well as in the regulation of bone turnover in adult women (Turner et al. 1994, Väänänen and Härkönen 1996). The mechanisms for the skeletal effects of estrogen have been subjected to intensive study, but are still incompletely understood. It is well documented that estrogen affects bone tissue by direct and indirect mechanisms, and that several of these effects are ER-mediated (Ciocca and Roig 1995). In addition to exerting direct effects on bone, estrogen improves the efficiency of intestinal calcium absorption and renal calcium reabsorption. Estrogen would thus seem not only to enhance BMD, but also to help the body accrue and retain the mineral necessary to strengthen the bone. Estrogen stimulates the synthesis of $1,25(OH)_2D$-vitamin in the kidney. Otherwise, there is little evidence that estrogen has any effect on the secretion of calciotropic hormones, although it apparently reduces PTH action on bone resorption (Prince 1994, Khosla et al. 1997).

An antiresorptive function characterises the influence of estrogen (Girasole et al. 1992, Turner et al. 1994, Väänänen and Härkönen 1996, Jilka 1998). Estrogen deficiency increases the rate of bone remodelling. Although bone formation is also enhanced, it is not able to compensate fully for markedly increased resorption. Thus, estrogen deficiency causes a rapid loss of bone mass, the most rapid changes being observed in trabecular bone (For

review, see Turner et al. 1994, Väänänen and Härkönen 1996). The interaction between estrogen and bone homeostasis may nonetheless be far more complex; the bone-conserving effect of estrogens has been found to be enhanced by calcium intake (Nieves et al. 1998) or by physical loading (Lanyon 1996, Notelovitz et al. 1991, Kohrt et al. 1995), although not consistently (Heikkinen et al. 1997).

Female sex hormone levels are cyclic in nature and physiologically integrated in the regular menstrual cycle. Early menarche is known to be associated with higher BMD (Ito et al. 1995, Johnell et al. 1995). In otherwise healthy women with normal menstruation cycles, low estrogen levels are associated with low BMD (Steinberg et al. 1989, Sowers et al. 1990, Sowers et al. 1998b). A low estradiol level during the follicular phase of the normal cycle is reported to be associated with monthly episodes of increased bone resorption (Chiu et al. 1999). Estrogen administration via oral contraceptives may abolish these estrogen fluctuations while establishing a relatively constant low estrogen level similar to normal early follicular phase levels. The role of oral contraceptive use relative to BMD during the reproductive years has not been resolved. According to recent reviews, the balance of evidence leans toward a positive association between oral contraceptive use and bone mass which, moreover, is directly related to the duration of oral contraceptive use and also shows dose response (Corson 1993, DeCherney 1996, Cromer 1999).

Menstrual irregularities, apparently representing states of transient hypoestrogenism, are related to reduced bone mass (Drinkwater et al. 1990, Armamento-Villareal et al. 1992). Hypoestrogenic amenorrhea, irrespective of the underlying specific cause, is associated with a reduction in bone mass (Cann et al. 1984, Davies et al. 1990, Haenggi et al. 1994, Ulrich et al. 1995, Park and Song 1995), and a high incidence of fractures (Davies et al. 1990). The degree of osteopenia in amenorrheic women is, however, highly variable (Schachter and Shoham 1994, Park and Song 1995, Miller and Klibanski 1999), and may be confounded by other underlying causes, including hyperprolactinemia (Klibanski et al. 1980, Biller et al. 1992), excessive exercise (Drinkwater et al. 1984), anorexia nervosa (Rigotti et al. 1991,

Grinspoon et al. 1999) and premature ovarian failure due to diverse reasons (Metka et al. 1992, Anasti et al. 1998).

An inverse correlation between bone mass and duration of amenorrhea has been reported in many studies (Klibanski and Greenspan 1986, Davies et al. 1990, Biller et al. 1991, Rigotti et al. 1991, Grinspoon et al. 1999), not however consistently (Cann et al. 1984, Schlechte et al. 1987). The amenorrheic bone loss is most distinct in trabecular bone (Cann et al. 1984, Drinkwater et al. 1984, Schlechte et al. 1987, Haenggi et al. 1994, Keen and Drinkwater 1997). The most rapid bone loss appears to occur at the onset of amenorrhea (Klibanski and Greenspan 1986, Davies et al. 1990, Biller et al.1991, Metka et al. 1992, Haenggi et al. 1994, Anasti et al. 1998). Although appropriate treatment apparently enhances BMD, the detrimental effects of amenorrhea may be irreversible (Drinkwater et al. 1986, Klibanski and Greenspan 1986, Biller et al. 1992, Gulekli et al. 1994, Keen and Drinkwater 1997), or limited to specific sites (Drinkwater et al. 1990, Haenggi et al. 1994). The partial recovery of BMD may be due to a too long duration of the preceding amenorrhea (Biller et al. 1991), low body weight (Drinkwater et al. 1990, Biller et al. 1991), underlying diagnosis of amenorrhea (Drinkwater et al. 1986, Gulekli et al. 1994), ovulatory disturbances despite regular menses (Prior et al. 1991) or some limit the bone can be regained following the menses (Drinkwater et al. 1990). No relation of recovery to the duration of amenorrhea has been found (Drinkwater et al. 1986), but the gain in BMD has been reported to be the better the more severe the osteopenia (Gulekli et al. 1994, Haenggi et al. 1994).

In the majority (65% to 75%) of amenorrheic women, estrogen deficiency has been hypothesised as the factor through which amenorrhea affects bone mass (See reviews by Schachter and Shoham 1994, Miller and Klibanski 1999). Although osteopenia in women with secondary amenorrhea caused by hyperprolactinemia has sometimes been attributed to the hyperprolactinemia itself (Schlechte et al. 1983, Schlechte et al. 1992), current thinking conceives the degree of estrogen deficiency to be most important (Klibanski et al. 1988, Biller et al. 1992). The distinction between amenorrhea due to estrogen deficiency and that due to other causes is important in considering the long-term implications of the disorder

(Schlechte et al. 1992, Schachter and Shoman 1994). Whether the resumption of normal menstruation in treated women with temporary but substantial amenorrhea is sufficient to counterbalance the negative effect of transient hypoestrogenism within the fertile period of life is unknown, but an increase in circulating estradiol coincident with resumption of menses is regarded as the primary factor in reversing bone loss (Drinkwater et al. 1986, Klibanski and Greenspan 1986, Biller et al. 1992).

It is of note, that factors besides estrogen deficiency may also modulate the degree of osteopenia in amenorrheic premenopausal women (Schlechte et al. 1987). In particular, body composition may make a significant contribution to the pathogenesis of amenorrheic bone loss (Klibanski and Greenspan 1986, Drinkwater et al. 1990, Biller et al. 1991, Biller et al. 1992, Grinspoon et al. 1999). The data from these studies suggest the critical importance of nutritional factors among premenopausal women with amenorrhea and estrogen-deficient bone loss. Moreover, the above-mentioned conditions associated with amenorrhea are also associated with a variety of additional neuroendocrine and/ or psychosomatic dysfunctions which apparently also have a direct impact on bone (Schachter and Shoman 1994).

3. Reproduction and bone

3.1 Hormonal control of pregnancy and lactation

After the sixth week of pregnancy the placenta takes over the function of the corpus luteum of pregnancy and produces progressively increasing levels of hormones, including estrogens, progesterone, prolactin and placental lactogen. This leads to the suppression of pituitary gonadotropin secretion characteristics of pregnancy.

After the birth of the baby, the hypothalamus-pituitary-ovarian axis takes its time to recover from the suppressive effects of pregnancy steroids. At term, placental steroids decline rapidly over 2 to 3 days postpartum to an undetectable level, plasma concentrations of

prolactin are very high, and both follicle-stimulating hormone (FSH) and luteinising hormone (LH) are undetectable during the first week postpartum (Dada and Laditan 1982, Shaaban et al. 1987, Kremer et al. 1994). In the absence of lactation, plasma prolactin concentrations decline to normal limits over the first 30 days (Howie et al. 1982), FSH and LH synthesis is resumed around day 10 (Kremer et al. 1994) and the hypothalamic-pituitary-ovarian axis recovers over a 40-day period, resulting in the first menstruation some 2 months postpartum (Howie et al. 1982, Moran et al. 1994, reviewed by McNeilly 1993).

Breastfeeding prolongs the suppression of the ovarian cyclicity postpartum and results in a period of lactational amenorrhea (Howie et al. 1982, Heinig et al. 1994, Moran et al. 1994). A notable feature of this period is its highly variable duration among different communities, although most breastfeeding mothers start menstruating during the first postpartum year (Shaaban et al. 1987, Lewis et al. 1991, Heining et al. 1994, Moran et al. 1994, Vestermark et al. 1994). Approximately fifty per cent resume menstruation while they are breastfeeding in spite of a high suckling frequency (Diaz 1989, Kurz et al. 1993, Campino et al. 1994, Vestermark et al. 1994), and there is 6% to 15% probability of menstruation even during full breastfeeding, i.e., baby receiving breast-milk alone (Lewis et al. 1991) or less than 120 ml/day (Heinig et al. 1994) or 146 KJ/day (Kurz et al. 1993) of other nutrients.

Lactational amenorrhea is characterised by elevated basal plasma prolactin concentrations and increased prolactin secretion in response to suckling (Howie et al. 1982, Diaz et al. 1989). Plasma concentrations of prolactin remain elevated during the first 3 to 4 months postpartum, declining with time in parallel with the decline in suckling activity (Dada and Laditan 1982, Howie et al. 1982, Shaaban et al. 1987, Diaz et al. 1989, Diaz et al. 1991, Nunley et al. 1991, Burger et al. 1994).

Plasma concentrations of FSH reach a level comparable to the upper part of the normal follicular range in 4 to 8 weeks (Dada and Laditan 1982, Kremer et al. 1990, Burger et al. 1994). LH remains undetectable during the first 3 weeks, and slowly increases thereafter to levels comparable with the lower part of the normal follicular range (Kremer et al. 1990,

Burger et al. 1994), while its pulsatile release remains inadequate (Kremer et al. 1991, Nunley et al. 1991). FSH secretion recovers earlier than LH secretion in the postpartum period (Diaz et al. 1995, Zinaman et al. 1995), leading to a period with a high FSH to LH ratio and follicular inactivity (Kremer et al. 1990). Thus, two patterns of pituitary secretion are distinguished: a completely suppressed pulsatile LH secretion together with low FSH concentration during the first 2 weeks and a period with partially suppressed pulsatile LH secretion together with relatively high FSH concentrations during the following weeks (Kremer et al. 1990, Kremer et al. 1991). Despite the relatively high FSH levels during lactation, hardly any follicular growth or any production of estradiol and progesterone occur in the absence of adequate LH secretion (Kremer et al. 1994).

Estrogen levels are thus suppressed in lactating women (Kremer et al. 1990, Kremer et al. 1991, Diaz et al. 1992, Caird et al. 1994, Dobnig et al. 1995, Lopez et al. 1996, Sowers et al. 1996, Krebs et al. 1997, Sowers et al. 1998c), even to a lower level than that of the early follicular range. Breastfeeding status as well as months since parturition have been reported to be significantly associated with estrogen levels (Diaz et al. 1995, Sowers et al. 1996). Apparently hypoestrogenemia remains present for at least 3 months postpartum, but no longer after 5-6 months lactation (Shaaban et al. 1987, Burger et al. 1994, Dobnig et al. 1995). In some women transient increases in ovarian estrogen secretion occur, indicating a modicum of follicular development, but ovulation rarely occurs (Shaaban et al. 1987, Diaz et al. 1991, Lewis et al. 1991). The first menstruation is regarded as a good indicator of the recovery of ovulatory ovarian function (Howie et al. 1982, Diaz 1989, Diaz et al. 1992, reviewed by Zarate and Canales 1987, McNeilly 1993, McNeilly et al. 1994).

The endocrine mechanisms responsible for the suppression of the hypothalamus-pituitary-ovarian axis are not yet fully understood (See for reviews Zarate and Canales 1987, McNeilly 1993, McNeilly et al. 1994). A central factor underlying the duration of PPA seems to be the suckling stimulus of the baby. Intense suckling can delay the resumption of menses and ovulation for even 1 to 3 years (Shaaban et al. 1987, Diaz et al. 1989, Lewis et al. 1991). Suckling appears to suppress the normal pattern of pulsatile release of

gonadotrophin-releasing hormone (Kremer et al. 1991, Zinaman et al. 1995) and hence prevents the normal growth of follicles. The normal positive feedback effect of estrogen on LH release is abolished, and estradiol exerts an enhanced negative feedback effect on both LH and FSH. Thus, while suckling continues, any follicle which starts to develop and secrete estradiol will further inhibit LH release and therefore stop growing. There is no clear role for prolactin per se in the suppression of ovarian activity postpartum (Diaz et al. 1988, Zinaman et al. 1995). As suckling declines, the pulsatile pattern of LH returns to normal, sensitivity to estrogen negative feedback declines, follicle growth can continue and ovulation will occur. Thus, ovarian activity and ovulation usually proceed as a result of a decline in suckling frequency or in the duration of suckling (Howie et al. 1982, Diaz 1989, Rosner and Schulman 1990), this possibly precipitated by the introduction of supplementary food (Howie et al. 1982, Diaz 1989, Kurz et al. 1993) and termination of night feeding (Howie et al. 1982, Diaz 1989, Rosner and Schulman 1990, Heinig et al. 1994).

However, the duration of PPA is only partially explained by the duration of breastfeeding or the suckling pattern (Diaz 1989, Lewis et al. 1991, Heinig et al. 1994), and it varies greatly even among homogeneous groups with apparently comparable nursing practice (Diaz et al. 1991, Diaz et al. 1995). This suggests differences between nursing women either in suckling stimulus intensity or in the sensitivity of the mother to the inhibitory effects of this stimulus on the release of the pituitary gonadotropins and the consequent stimulation of ovarian activity (Diaz 1989, Diaz et al. 1989, Diaz et al. 1991, Campino et al. 1994). Also, maternal age (Diaz 1989, Kurz et al. 1993, Moran et al. 1994) and parity (Diaz 1989, Kurz et al. 1993, Heining et al. 1994) have been reported to prolong the length of PPA. No clear relationship between maternal nutritional status and PPA has been reported (Diaz 1989, Diaz et al. 1991, Lewis et al. 1991, Kurz et al. 1993).

3.2 Calcium metabolism

Calcium metabolism during reproduction differs from that of the normal state in fundamental ways, although some of the relevant data are contradictory (reviewed by

Kovacs and Kronenberg 1998, Power et al. 1999, Prentice 2000). During pregnancy the fetal calcium needs are primarily met by enhanced intestinal calcium absorption (Kent et al. 1991a, Cross et al. 1995a, Ritchie et al. 1998). Immediately after delivery, absorption efficiency falls toward nonpregnant levels and remains at this relatively low level throughout lactation (Kent et al. 1991a, Kent et al. 1993, Cross et al. 1995a, Ritchie et al. 1998), even in women with different calcium intakes (Specker et al. 1994, Kalkwarf et al. 1996). Urinary calcium, which is high during pregnancy (Dahlman et al. 1994, Seely et al. 1997) falls at the same time and remains low throughout lactation and for several months postweaning (Kent et al. 1990, Kent et al. 1991b, Kent et al. 1993, Specker et al. 1994, Prentice et al. 1995, Ritchie et al. 1998, Kalkwarf et al. 1999). Not all data, however, indicate that renal calcium reabsorption is increased during lactation (Cross et al. 1995b, Krebs et al. 1997).

PTH levels are typically normal during pregnancy (Saggese et al. 1991, Dahlman et al. 1994, Gallacher et al. 1994, Cross et al. 1995a, Seely et al. 1997, Ritchie et al. 1998). Neither are they elevated in early lactation or mid-lactation (Kent et al. 1990, Cross et al. 1995b, Ritchie et al. 1998, Sowers et al. 1998c, Prentice et al. 1998), when the PTH concentration may be even below the levels of nonlactating women (Dobnig et al. 1995, Krebs et al. 1997, Kalkwarf et al. 1996, Kalkwarf et al. 1999). However, several studies have reported increasing concentrations during and after weaning (Kent et al. 1990, Specker et al. 1991, Verhaeghe and Bouillon 1992, Cross et al. 1995a,b, Kalkwarf et al. 1996, Kalkwarf et al. 1999, Prentice et al. 1998, Sowers et al. 1998c). The concentration of PTH-related peptide (PTHrP) is elevated during pregnancy (Gallagher et al. 1994, Kovacs and Kronenberg 1998) and lactation (Dobnig et al. 1995, Sowers et al. 1998c). The concentration of calcitonin apparently remains within normal limits during pregnancy (Cross et al. 1995a, Ritchie et al. 1998) and lactation (Chan et al. 1987, Krebs et al. 1997, Ritchie et al. 1998). In contrast, serum $1,25(OH)_2D$ levels rise early in pregnancy, being double the normal range, and this high level is maintained until term (Wilson et al. 1990, Verhaeghe and Bouillon 1992, Cross et al. 1995a, Seely et al. 1997, Ritchie et al. 1998), whereas $1,25(OH)_2D$ is not elevated during lactation (Kent et al. 1990, Cross et al. 1995a,b, Specker et al. 1991, Specker et al. 1994, Kalkwarf et al. 1996, Ritchie et al. 1998). With time from partus, however,

1,25(OH)$_2$D concentrations show gradually increasing levels (Wilson et al. 1990, Krebs et al. 1997, Prentice et al. 1998, Kalkwarf et al. 1999), albeit again not in all studies (Sowers et al. 1998c).

3.3 Bone turnover

As during pregnancy (Rodin et al. 1989, Cross et al. 1995a, Naylor et al. 2000), bone turnover during the postpartum period is in an elevated state (Cross et al. 1995b, Prentice et al. 1998). Breastfeeding women have a higher bone turnover than bottle-feeding women (Kent et al. 1990, Zinaman et al. 1990, Sowers et al. 1993, Caird et al. 1994, Dobnig et al. 1995, Affinito et al. 1996, Lopez et al. 1996, Krebs et al. 1997, Kalkwarf et al. 1999). The duration of lactation is an essential determinant of bone turnover (Sowers et al. 1995a, Yamaga et al. 1996), and women with longer lactation have higher formation marker levels for longer periods (Sowers et al. 1995a). It has been postulated that the bone loss during early lactation is attributable to a high bone resorption to formation ratio (Kent et al. 1990, Sowers et al. 1993, Dobnig et al. 1995, Prentice et al. 1998). During the postweaning period the bone resorption rate has reverted to virtually normal while the bone formation rate remains elevated (Kent et al. 1990, Dobnig et al. 1995, Ritchie et al. 1998, Kalkwarf et al. 1999). Typical bone turnover markers measured in reproduction include circulating osteocalcin (OC), bone-specific alkaline phosphatase (BAP) and carboxy-terminal propeptide of type I procollagen (PICP) as indicators of bone formation, and urinary collagen cross-links and serum tartrate-resistant acid phosphatase (TRAP) as indicators of bone resorption.

The OC concentration is lower throughout pregnancy than before conception (Cross et al. 1995a, Ritchie et al. 1998), although late gestation concentrations are higher than those in early pregnancy (Cross et al. 1995a, Yamaga et al. 1996, Ritchie et al. 1998). This may be due to uptake of OC by the placenta (Salle et al. 2000). Studies have consistently shown that the serum concentration of OC tends to be comparable to normal nonpregnant control for some time after delivery (Sowers et al. 1993, Dobnig et al. 1995, Sowers et al. 1995a,

Affinito et al. 1996, Krebs et al. 1997, Prentice et al. 1998). Only a few studies have reported elevated OC levels already at partus (Lopez et al. 1996, Yamaga et al. 1996). In general thereafter, OC have been reported to rise up to 3 to 4 months (Cross et al. 1995a, Sowers et al. 1995, Yamaga et al. 1996, Krebs et al. 1997, Prentice et al. 1998, Kalkwarf et al. 1999) or to 6 months (Sowers et al. 1993, Dopnig et al. 1995, Caird et al. 1994, Affinito et al. 1996) during lactation, and subsequently to fall to baseline levels by 2 to 6 months postweaning (Kent et al. 1990, Krebs et al. 1997) or 12 to 18 months postpartum (Sowers et al. 1995a, Affinito et al. 1996).

BAP has been reported to increase gradually during pregnancy, resulting in elevated levels at partus (Rodin et al. 1989, Cross et al. 1995a, Naylor et al. 2000) and showing a virtually unchanged or slightly decreasing trend thereafter (Cross et al. 1995a, Sowers et al. 1995a, Prentice et al. 1998). PICP levels have been reported to be elevated during the third trimester of pregnancy (Cross et al. 1995a) and during lactation (Cross et al. 1995a, Dobnig et al. 1995).

Bone resorption markers are most markedly elevated at partus (Cross et al. 1995a, Krebs et al. 1997, Prentice et al. 1998, Kalkwarf et al. 1999, Naylor et al. 2000), while the pattern thereafter is somewhat obscure. The bone resorption markers have been reported either to decrease during the first 3 to 6 months postpartum (Cross et al. 1995a, Lopez et al. 1996, Krebs et al. 1997, Prentice et al. 1998, Kalkwarf et al. 1999, Naylor et al. 2000), or to increase (Ritchie et al. 1998), or first to increase for 1 to 3 months and then to decrease (Dobnig et al. 1995, Yamaga et al. 1996).

3.4 Bone mineral density (BMD)

Severe bone loss leading to idiopathic osteoporosis and fracture in young women is a well recognised but rare complication of pregnancy (reviewed by Khovidhunkit and Epstein 1996). The clinical course of this condition is variable. Most patients sustain vertebral fractures, usually late in pregnancy or shortly after the delivery of their first child. In most

the symptoms resolve spontaneously after delivery, abortion or cessation of lactation, while some continue to have debilitating pain for several years. The condition tends not to recur in subsequent pregnancies. The syndrome appears to be clinically distinct from transient osteoporosis of the hip, another syndrome occurring during pregnancy, which typically appears earlier, may be localised in one hip only, and proceeds almost always without fracture (Brooks et al. 1990, Funk et al. 1995). It is unclear whether pregnancy is the causative factor in these syndromes or merely the highlighting event. Although lactation cannot be the etiological factor in most women, it may lead to a further reduction in BMD.

Prospective studies including pre- and postpregnancy measurements are sparse, and the effect of pregnancy on BMD is as yet quite inconclusive (Sowers 1996a, Sowers 1996b). On one hand, no change in the BMD of the radius (Cross et al. 1995a), the proximal femur (Sowers et al. 1991b) or the lumbar spine (Ritchie et al. 1998) has been reported. On the other, some 4% mean losses in lumbar (Drinkwater and Chesnut 1991, Black et al. 2000, Naylor et al. 2000), and femoral and radial BMD (Drinkwater and Chesnut 1991, Black et al. 2000) have been observed.

The recent prospective studies of postpartum women including measurements of the axial skeleton have been carried out with DXA (Table 1), an approach which is the present method-of-choice for clinical osteoporosis and bone research (Genant 1998). Earlier reports of prospective changes in bone mass have mainly rested on single photon absorptiometry (SPA) and dual photon absorptiometry (DPA) technology (Atkinson and West 1970, Lamke et al. 1977, Chan et al. 1987, Hayslip et al. 1989, Kent et al. 1990, Drinkwater and Chesnut 1991, Kent et al. 1993, Cross et al. 1995a, Prentice et al. 1995, Affinito et al. 1996, Krebs et al. 1997), and by reason of this difference in technique these studies are not addressed here. According to DXA studies, it is obvious that the mean decrease in BMD is some 4% to 7% in the spine and femoral neck during the first 3 and 6 months of lactation. Also total bone mineral content is reported to decline during lactation (Kalkwarf and Specker 1995, Kolthoff et al. 1998, Ritchie et al. 1998, Laskey and Prentice 1999, Hopkinson et al. 2000). However, the lactation-associated decrease in BMD seems to be temporary and to be

reversed when breastfeeding declines and ceases, and when menstruation resumes (Lopez et al. 1996, Laskey and Prentice 1999, Polatti et al. 1999). The recovery is apparent even in women who become pregnant during lactation (Sowers et al. 1995b, Laskey and Prentice 1997).

Prior to the initiation of the present work only two studies using SPA and/or DPA (Drinkwater and Chesnut 1991, Cross et al. 1995a) had also included prepregnancy BMD measurements. Compared to prepregnancy, there seemed to be no difference in bone mass in the lumbar spine postmenses or postweaning (Cross et al. 1995a). Drinkwater and Chesnut confined BMD measurements to 6 months of lactation, during which time the BMD of the radius and spine returned to prepregnancy levels while femoral BMD continued to decline.

The net effect of reproduction on bone mass is largely unresolved (Eisman 1998). Parity has been found to be related to a reduced fracture risk later in life (Wyshak 1981, Paganini-Hill et al. 1991, Kelsey et al. 1992, Hoffman et al. 1993, Tuppurainen et al. 1993), but also no association has been reported (Kreiger et al. 1982, Alderman et al. 1986, Torgenson et al. 1996). However, current epidemiological evidence seems fairly consistent with regard to the protective effect of a lactation history against spinal (Aloia et al. 1985), hip (Kreiger et al. 1982, Alderman et al. 1986, Cumming and Klineberg 1993) and arm (Kelsey et al. 1992) fractures.

According to retrospective studies resting chiefly on the SPA/DPA technique, the effect of parity on BMD is even more conflicting. This is not surprising due to large individual variation in BMD, variable study designs, variable data and definition of lactation and several confusing factors related to time. No association has been found (Armamento-Villareal et al. 1992, Kritz-Silverstein et al. 1992, Melton et al. 1993, Bauer et al. 1993, Berning et al. 1993, Bererhi et al. 1996, Sinigaglia et al. 1996), but there is also evidence for a positive correlation (Fox et al. 1993) and that nulliparity is associated with lower BMD (Sowers et al. 1992b). Some studies, again, have shown that parity is associated with low

BMD (Lissner et al. 1991, Ghannam et al. 1999) or its effect is skeletal site dependent (Hreshchyshyn et al. 1988).

Studies of lactation with respect to its impact on BMD are highly inconsistent (reviewed by Sowers 1996a, Sowers 1996b, Tudor-Locke and McColl 2000). According to the literature, the effect of lactation on postmenopausal bone mass is either negligible (Wasnich et al. 1983, Kritz-Silverstein et al. 1992, Fox et al. 1993, Bauer et al. 1993, Sinigaglia et al. 1996) or positive (Aloia et al. 1983, Berning et al. 1993). In contrast, where pre- or perimenopausal bone mass is concerned, previous retrospective studies have observed either a negative association (Goldsmith and Johnston 1975, Lissner et al. 1991, Ghannam et al. 1999) or no association (Sowers et al. 1985, Koetting and Wardlaw 1988, Stevenson et al. 1989, Cox et al. 1991, Sowers et al. 1992b, Armamento-Villareal et al. 1992, Melton et al. 1993) with duration of lactation. Some studies have suggested site specificity, indicating that in sites containing primarily trabecular bone lactation is associated with high (Hreshchyshyn et al. 1988, Feldblum et al. 1992) or low BMD (Wardlaw and Pike 1986).

TABLE 1. Longitudinal studies of site-specific postpartum BMD with DXA.

Study	Times of measurement	Site(s)	Sample size	Mean dietary calcium/ day	Duration of lactation	Duration of PPA	Findings
Sowers 1993	2 wk, 2, 4, 6, 12 mo	Spine Femoral neck	20 short-term (0-1 mo) BF 26 mid-term (2-5 mo) BF 64 long-term (>6 mo) BF	900 mg 1200 mg 1600 mg	BF = 2/3 of infant need	NA	Loss of 5.1%/ 6 mo in spine, 4.8% in femur, partial recovery in long-term BF No change in others
Caird 1994	4 wk, 6, 12 mo	Spine	9 BF 9 oral contraception BF 10 FF controls	≥800 mg	≥6 mo	Median 36 wk 41 wk 6.3 wk	Loss of 4.9%/ 6 mo, full recovery in BF Loss of 3%/ 6 mo, full recovery in oral contraception BF Gain of 4.3%/ 12 mo in FF
Cross 1995b	2wk, 3 mo, 3 mo pw	Forearm Spine	7 ca suppl BF 8 placebo BF	1100 mg	Fully BF at 6 wk in 100%, at 3 mo in 80%, at 6 mo in 40%. Mean BF 8.3 vs 6.8 mo	9.8 mo	Loss of 4.3%/ 3 mo in placebo BF and 6.3% in ca BF in spine. Partial recovery. Gain of 5.7%/ 3 mo in ultradistal forearm. Loss of 6 to 11 % pw in forearm.
Kalkwarf 1995	a) 2, 14, 26 wk b) 24, 36, 48 wk	Radius Spine	a) 65 BF 48 FF controls b) 40 BF 43 FF controls	800 mg	a) Fully BF 4.7 mo b) ≥6 mo fully BF Fully BF = at most 1 formula/day	a) ≥6 mo in 70% of BF, 8 wk in FF b) 32 wk in BF, ≤24 wk in 98% of FF	a) Loss of 3.9%/ 3 mo, no further loss/ 6 mo in spine in BF Gain of 1.5% in FF No change in radius b) Gain of 5.5%/ 6 mo in spine in BF Gain of 1.8% in spine in FF No change in radius
Matsumoto 1995	5 d, 1, 3, 6, 12, 24 mo	Radius	11 BF 11 FF controls	NA	8.6 ± 0.9 mo	NA	Loss of 5.2%/ 6 mo and 11.7%/ 12 mo, partial recovery in BF No change in FF
Sowers 1995b	2 wk, 2, 4, 6, 12, 18 mo, 28 mo or 3wk after new pregn.	Spine Femoral neck	25 BF with a new pregn. 20 BF controls	1600 mg 1200 mg	Fully BF ≥6mo Fully BF =2/3 of infant need	7.4 ± 5.1 mo 7.7 ± 6.2 mo	Loss of 3.4% to 3.9%/ 6 mo in both groups. Full recovery, similar in both groups.
Lopez 1996	1, 6 mo, 6 mo pw	Spine Femoral neck	20 BF 22 non-preg controls	1000 mg 600 mg	Fully BF 6 mo (8.1 ± 2.2 mo) Fully BF= not even water Total BF 13.4 ±4.6 mo	≥6 mo in 52% of BF	Loss of 5.5%/ 6 mo in spine, 3% in femur, recovery above baseline in BF No change in controls

TABLE 1 (continued)

Study	Times of measurement	Site(s)	Sample size	Mean dietary calcium/ day	Duration of lactation	Duration of PPA	Findings
Kalkwarf 1997	a) 2 wk, 3, 6 mo b) 6, 9, 12 mo	Radius Spine	a) 87 BF ca or placebo 81 FF ca or placebo b) 76 BF ca or placebo 82 FF ca or placebo	≤800 mg	a) Fully BF ≥6 mo b) Fully BF ≤8 mo (Fully BF= not more than 1 formula/day)	a) ≥6 mo 75% of BF b) ≥6 mo 85% of BF	a) Loss of 4.2%/ 3 mo ca, 4.9% placebo in spine with little further loss in 6 mo in BF Gain of 2.2%/ 6 mo ca, 0.4% placebo in spine in FF No change in radius b) Gain of 5.9% ca, 4.4% placebo in spine in BF Gain of 2.5% ca, 1.6% placebo in spine in FF
Laskey 1997	0.5, 3, 6 mo, 12 mo and 3 mo pw or 0.5 mo after a new pregn.	Spine Femoral neck	12 BF	1600 mg	≥3 mo	NA	Loss of 3.4%/ 3 mo and 3.1%/ 6 mo in spine Loss of 0.5%/ 3 mo and 2.8%/ 6 mo in femur Recovery, before and during subsequent pregnancy.
Honda 1998	5 d, 3, 6 mo	Spine	111 cases, grouped at 3 mo 69 BF, 42 FF, at 6 mo 61 BF, 32 FF at 3 mo 70 PPA, 41 menses, at 6 mo 51 PPA, 42 menses	NA	≥3 mo 62% ≥6 mo 55%	≥3 mo 63% ≥6 mo 56%	Loss of 4.9%/ 3 mo in spine and 5.9%/ 6 mo in BF or in PPA No change in FF
Kolthoff 1998	0, 3, 6, 12, 18 mo	Radius Spine Femoral neck	16 BF + PPA< 4 mo 26 BF + PPA 4-8 mo 17 BF +PPA> 8 mo	1600 mg 1200 mg 1300 mg	4.8 mo 8.8 mo 12.2 mo	6.1 mo	Loss of 5.2%/ 3 mo in spine, 5.3%/ 6 mo in femur, full recovery No change at radius
Laskey 1998	0.5, 3 mo	Spine Femoral neck Forearm	47 BF 11 FF 22 nonpregn. controls	1300 mg 1000 mg 1000 mg	≥3 mo Fully BF ≥2 mo in 89%, ≥3 mo in 79%	NA	Loss of 4%/ 3 mo in spine, 2% in femur in BF No change in radius, in FF, in controls
Ritchie 1998	2 mo, 5 mo pm	Spine	14 BF	1200 mg	12 ± 10 mo Fully BF ≥2 mo in 71%	8 ± 3 mo	Loss of 9%/ 2 mo at spine, full recovery to prepregnancy level
Laskey 1999	0.5, 3, 6, 12 mo (+ 3 mo pw)	Spine Femoral neck Forearm	59 BF 11 FF 22 nonpregn. controls	1500 mg 1100 mg 1000 mg	6.9 mo	5.6 mo	Loss of 3-4%/ 3 mo in spine, 4% in femur and 5%/ 6 mo in femur, recovery above baseline in spine (2.7%), partial in femur (-2%) in BF No change in FF, in controls
Polatti 1999	5 d, 3, 6, 12, 18 mo	Radius Spine	135 BF 139 ca suppl. BF 153 FF	NA	Fully BF 6 mo	≤5 mo 57% in BF, ≤5 mo 58% in ca BF, 35-55 d in FF	Loss of 4%/ 3 mo in spine and 2% in radius, recovery above baseline in spine (2%) and in radius (1%) in BF Gain of 2%/ 18 mo in spine and 1% in radius in FF

BF = breast-feeding; FF = formula-feeding; NA = not available

3.5 Factors underlying postpartum-related BMD changes

3.5.1 Lactation

Postpartum bone loss has been demonstrated only in breastfeeding women (Caird et al. 1994, Kalkwarf and Specker 1995, Matsumoto et al. 1995, Honda et al. 1998, Laskey et al. 1998, Polatti et al. 1999). In fact, according to some reports nonlactating postpartum women gain BMD in the lumbar spine (Caird et al. 1994, Kalkwarf and Specker 1995, Polatti et al. 1999). However, the effect of the duration of breastfeeding on bone changes is not clear. In one study, the magnitude and duration of bone loss was greater in women who breastfed for a longer time (Laskey and Prentice 1999). Breast-milk volume, but not breast-milk calcium concentration has been identified as a significant predictor for BMD change in the spine (Laskey et al. 1998).

Lactation-related spinal BMD losses have been reported to disappear by approximately 3 months postweaning regardless of the duration of breastfeeding (Cross et al. 1995b, Ritchie et al. 1998), but on the other hand, a longer duration of lactation has been reported to be associated with larger individual net losses in BMD 1 to 2 years after delivery (Kolthoff et al. 1998, Hopkinson et al. 2000).

3.5.2 Postpartum amenorrhea

In addition to lactation status, ovarian dysfunction is one of the potential factors behind bone loss during lactation (Honda et al. 1998). A shorter duration of PPA has been reported to be associated with a smaller loss of bone during lactation (Kalkwarf and Specker 1995, Sowers et al. 1996, Kalkwarf et al. 1997, Polatti et al. 1999). Earlier resumption of menses has been reported to be associated with a greater increase in BMD after weaning (Caird et al. 1994, Kalkwarf and Specker 1995, Kolthoff et al. 1998, Hopkinson et al. 2000), this however not consistently (Polatti et al. 1999). Changes after lactation have also been reported to be

largely independent of the duration of lactation or amenorrhea (Kalkwarf et al. 1997, Laskey and Prentice 1999).

3.5.3 Hormones

The evidence on classical calciotropic hormones suggests that they do not play a key role in bone metabolism accompanying human lactation (Specker et al. 1991, Dobnig et al. 1995, Cross et al. 1995b, Lopez et al. 1996, Sowers et al. 1998c). Neither does any study indicate that vitamin D requirements are any greater during lactation than during nonlactation (Specker 1994, Ghannam et al. 1999).

The role of the vital hormone of lactation, prolactin, is likewise not well established (Lopez et al. 1996, Sowers et al. 1996). As regards lactation, prolactin has suggested to have an indirect role in calcium metabolism through its interaction with estrogen in suppressing the hypothalamus-pituitary-axis (Zarate and Canales 1987). There is also some evidence that PTHrP, possibly modulated by prolactin, may regulate maternal bone metabolism during lactation, particularly during the early weeks after parturition (Dobnig et al. 1995, Sowers et al. 1996).

The bone loss and recovery during lactation and postweaning periods is very likely modulated, at least partially, by varying estrogen levels during these particular time periods (Hillman et al. 1981, Turner et al. 1994, Honda et al. 1998). In fact, positive associations of serum estradiol with BMD have been found in postpartum women (Krebs et al. 1997, Sowers et al. 1996), but this relationship seems to be somewhat controversial (Caird et al. 1994, Lopez et al. 1996, Ritchie et al. 1998).

3.5.4 Dietary calcium

The estimated milk production during the first 6 months of lactation average 700-750 g/day (Butte et al. 1984, Sadurskis et al. 1988, van Raaij et al. 1991, Prentice 1994). Approximately 200-300 mg calcium are secreted daily into breast-milk during full lactation (Laskey et al. 1990). However, a breastfeeding mother produces breast-milk with a characteristic concentration of calcium, and this concentration and the daily calcium output in breast-milk vary widely between mothers (Laskey et al. 1990). There is no convincing evidence that maternal calcium intake or use of calcium supplements (Prentice et al. 1995, Prentice 1998) influences the calcium concentration in breast-milk.

There is also a growing body of evidence that the skeletal response to lactation is independent of maternal calcium intake (Sowers et al. 1993, Kalkwarf and Specker 1995, Cross et al. 1995b, Lopez et al. 1996, Kolthoff et al. 1998, Laskey et al. 1998). In addition, some studies have indicated that calcium supplementation does not alter the pattern of bone mineral change during lactation, even in women accustomed to a very low calcium intake (Cross et al. 1995b, Prentice et al. 1995, Kalkwarf et al. 1997). On the other hand, the recovery of bone mineral after weaning may be influenced by dietary calcium (Kalkwarf et al. 1997).

Although, the current scientific data thus suggest that breastfeeding women need not consume extra calcium (Prentice 1997, Allen 1998, Abrams 1998), recommendations for calcium intakes during lactation differ around the world (i.e., 400 to 800 mg increase/ day) (Prentice 1994). The recommended intake of calcium for lactating women in Finland is 1200 mg/ day (Finnish Paediatric Association and The Mannerheim League for Child Welfare 1997).

3.5.5 Maternal weight

A link between lactation and weight loss has been proposed in view of the increased energy and nutrient expenditure needed for milk synthesis and secretion, but such a relation has been observed only in some studies (Brewer et al. 1989, Dewey et al. 1993, Kramer et al. 1993, Janney et al. 1997), and not consistently (Potter et al. 1991, Schauberger et al. 1992, Thorsdottir and Birgisdottir 1998). Neither maternal body size nor body composition have been reported to influence the quantity or quality of breast-milk (Butte et al. 1984, Dusdieker et al. 1994), but those who are overweight or obese may have difficulties in initiating or maintaining lactation (Rutishauser and Carlin 1992, Hilson et al. 1997). The recommendations concerning additional energy needs for exclusively breastfeeding women have been criticised as too high (Butte et al. 1984, Sadurskis et al. 1988, Brewer et al. 1989, van Raaij et al. 1991, Todd and Parnell 1994). However, mothers with a lower body fat content may have greater dietary energy requirements (Butte et al. 1984, Winkvist and Rasmussen 1999). The current recommendation on energy needs for lactating women in Finland is 500-1000 kcal (2.1-4.2 MJ)/day greater than those of nonlactating women (Finnish Paediatric Association and The Mannerheim League for Child Welfare 1997).

Women worldwide generally lose weight during lactation (Winkvist and Rasmussen 1999), and this seems to be mainly explained by body fat losses (Sadurskis et al. 1988, van Raaij et al. 1991, Motil et al. 1998). The average weight loss during the first 6 months postpartum is some 8 to 13 kg (Brewer et al. 1989, Schauberger et al. 1992, Kramer et al. 1993), although very modest losses, 3-5 kg, have also been reported (Butte et al. 1984, Sadurskis et al. 1988, van Raaij et al. 1991, Rutishauser and Carlin 1992, Dewey et al. 1993, Motil et al. 1998). Lactation-associated bone loss cannot be explained by differences in body size (Sowers et al. 1993, Lopez et al. 1996, Laskey and Prentice 1999) or by weight changes (Kolthoff et al. 1998, Laskey et al. 1998).

3.5.6 Other factors

The maternal age (Sowers et al. 1993, Kolthoff et al. 1998, Laskey and Prentice 1999) or parity (Lopez et al. 1996, Laskey and Prentice 1999) has apparently no effect on BMD changes. In only one study did parity predict net bone gains in lactating women (Hopkinson et al. 2000). Nor can bone loss be explained by differences in physical activity (Sowers et al. 1993). The small amounts of gestagen in contraceptive pills may protect against bone loss during lactation (Caird et al. 1994), but such a finding has not been consistent (Laskey et al. 1998, Ritchie et al. 1998).

4. Summary

Although osteoporosis and associated fracture risk concern primarily older persons, a predisposition to osteoporosis may be generated in part during young adulthood. To reduce future fracture risk, efforts should focus on maximising and maintaining peak bone mass already in premenopausal women. There is some controversy as to the course of premenopausal bone mass, but the apparent fact is that most of the bone mass in multiple skeletal regions is accumulated by late adolescence, and that thereafter hardly any substantial premenopausal bone loss occurs under normal conditions.

Estrogen deficiency and calcium metabolism clearly play major roles in the development of osteoporosis. Pregnancy and lactation are phases in a woman's reproductive life associated with profound changes in calcium metabolism and hormone levels. The past decade has greatly broadened our understanding of reproduction and bone. Compensatory mechanisms probably come into play early in pregnancy, these comprising an increase in the circulating concentration of $1,25(OH)_2D$ and intestinal calcium absorption. However, the precise impact of pregnancy on BMD is currently controversial and less well studied. Besides hypoestrogenemia the extra calcium requirements in lactation are far more pronounced than

those during pregnancy. Some calcium conservation may be achieved through a reduction in urinary calcium, but this mechanism is not sufficient to prevent bone loss. Bone turnover during lactation is sharply accelerated as judged from biochemical markers, but the detailed patterns remain to be established. According to published studies there is a consensus that BMD is to some extent reduced during lactation, but also recovers rapidly to post-pregnancy values after weaning. However, the long-term relations between osteoporosis and parity or lactation are for the present unknown.

It is unclear whether the length of lactation, length of postpartum amenorrhea, or simply the time elapsed since childbirth are important for loss and recovery in BMD. It is obvious, again, that the lactation-related loss in BMD is independent of dietary calcium intake and calcium supplementation, but that additional calcium may have a small beneficial effect on the recovery in BMD after weaning. The effects of other underlying factors remain more obscure. Alterations in calcium homeostasis during lactation are apparently not driven by changes in levels of calciotropic hormones. Instead, these changes have been held to be determined by the combined effects of low estrogen concentrations and a high concentration of PTHrH from the breast possibly driven by prolactin; however, the current data are not adequate to draw precise conclusions regarding the mechanisms underlying postpartum-related BMD changes.

AIMS OF THE STUDY

The aims of this study were:

1. to evaluate changes in BMD during reproduction with a novel physiology-related study design (I),

2. to determine the proportion of women who do not show a loss or recovery in BMD postpartum (II),

3. to explore whether mother's genotype (VDR and ER polymorphisms) modulates the anticipated changes in BMD postpartum, and if so, to what extent (III),

4. to determine factors accounting for the anticipated changes in BMD postpartum (IV), and

5. to determine the factors which essentially modulate bone turnover after parturition and subsequently contribute to incident changes in bone mineral (V).

MATERIALS AND METHODS

1. Study subjects

Basic study group

All healthy adult women (n=119) registered at the mothers' health care unit in Kangasala Health Center, southern Finland, due to pregnancy during a one-year period from November 1992 to October 1993 were asked to participate in this study. Also, during a concurrent 6-month period, healthy women who were definitely planning pregnancy in the very near future were asked to participate in the study by the information distributed to mothers' health care units, health center doctors and nurses in Kangasala Health Center, and by two items in the local newspaper.

Initially 85 women volunteered, and 5 of them enrolled before pregnancy. Seven withdrew from the study during the pregnancy. Study measurements were performed on 78 women after delivery, on 61 women soon after the resumption of menses, and on 45 women one year thereafter. Reasons for withdrawal after delivery due to study participants were a new pregnancy (n=15), loss of interest (n=13), a disease (n=2), and a change in residence (n=3). Moreover, two women were excluded from the data analysis due to concurrent thyroxin and cortisone treatment during the study period. Thus, the study samples comprised 5 women before pregnancy, 76 women after delivery, 59 women after the resumption of menstruation and 43 women one year thereafter. It may however be noted that the baseline anthropometry or BMD of all above mentioned dropouts did not differ from those in subjects followed till the end of the study, but the dropouts were somewhat younger (29.2 years versus 31.3 years).

The baseline clinical data of the basic 76 mothers are presented in Table 2. Thirty-one mothers were primiparas. Forty of the infants were males and 36 females. Consumption of calcium and vitamin D supplementation was extremely rare, only one woman used calcium,

two vitamin D and eleven multivitamin supplementation occasionally. Information on this aspect was not obtained from five women. All mothers were ambulant with no history of excessive exercise or caffeine or alcohol consumption, nor periods of immobilisation. Smoking data were not obtained from 3 women, 44 women had never smoked, 20 had stopped smoking, 4 smoked occasionally and 5 daily (mean 5 cigarettes/day).

The study protocol was approved by the Ethics Committee of Tampere University Hospital and the UKK Institute in Tampere, and written informed consent was obtained from the subjects.

TABLE 2. Baseline characteristics of study subjects (n=76) after delivery.

	Mean	SD	Range
Age (years)	30.4	4.3	22.5 – 42.0
Weight (kg)	69.3	9.9	51.0 – 99.2
Height (cm)	166.0	5.9	154.5 – 182.0
BMI (kg/cm^2)	25.1	3.4	19.6 – 35.3
Parity	0.9	1.0	0 – 4
Duration of previous lactation (of multiparas; months)	10.5	7.1	0.5 – 33.0
Age at menarche (years)	12.8	1.5	10.0 – 17.0
Duration of pregnancy (weeks)	40.0	1.5	34.5 – 42.3
Child weight (g)	3600	530	2370 – 4720
Child height (cm)	51.1	2.3	45.0 – 56.0
Dietary calcium intake (mg/day) n=59	1220	510	400 – 3400
BMD in lumbar spine (g/cm^2)	1.039	0.090	0.852 – 1.201
BMD in femoral neck (g/cm^2)	0.914	0.097	0.684 – 1.145
BMD in distal radius (g/cm^2)	0.359	0.040	0.253 – 0.468
t-score of lumbar spine*	+0.05	0.90	−1.82 – +1.67
t-score of femoral neck*	−0.25	0.90	−2.38 – +1.89
t-score of distal radius*	+0.19	1.0	−2.45 – +2.93

*T-scores were calculated using the data of healthy 20 to 40 –year old females as reference values, i.e., 1.034 (0.100) g/cm^2 for the lumbar spine; 0.941 (0.108) g/cm^2 for the femoral neck; and 0.351 (0.040) g/cm^2 for the distal radius (adapted from the study by Haapasalo et al. 1996).

Selection criteria for the studies

Study I comprised the five volunteers who enrolled in the study before the pregnancy and remained involved till the end of the follow-up. At the time of recruitment, the age of these women was 27.0 (SD 2.6) years, weight 63.7 (10.5) kg and height 171.1 (4.0) cm. One woman had previously had one two-year old child. At recruitment, BMD data were in line with age-matched reference data (see footnote to Table 2), the mean t-scores being 0.7 (range –0.4 to 1.9) for the lumbar spine, 0.0 (–1.3 to 1.8) for the femoral neck and 0.7 (–0.2 to 1.5) for the distal radius.

Study II assessed changes in BMD during PPA, including the mothers who continued in the study until the resumption of menses (n=59), or until the end of the follow-up one year thereafter (n=43).

Study III comprised genotyping data of those 43 mothers who completed the entire follow-up period.

Study IV focused on a search for factors best explaining changes in BMD during postpartum amenorrhea and the subsequent follow-up period after resumed menstruation. For this reason, one woman was excluded due to missing data on menstruation, and one due to violation of study protocol i.e., a grossly delayed BMD measurement. Thus, paper IV described data on the 41 mothers who completed the entire follow-up period.

Study V was carried out specifically to assess the effect of PPA and resumption of menstruation on serum female sex hormone levels and on a variety of serum markers of bone turnover. One woman was excluded due to missing data on menstruation, and one due to a grossly delayed BMD measurement, as mentioned above. Study V includes 32 mothers whose entire biochemical laboratory data were obtained and who adhered to the study protocol and completed the entire follow-up period. The subjects and purposes of studies I-V are presented in Figure 1.
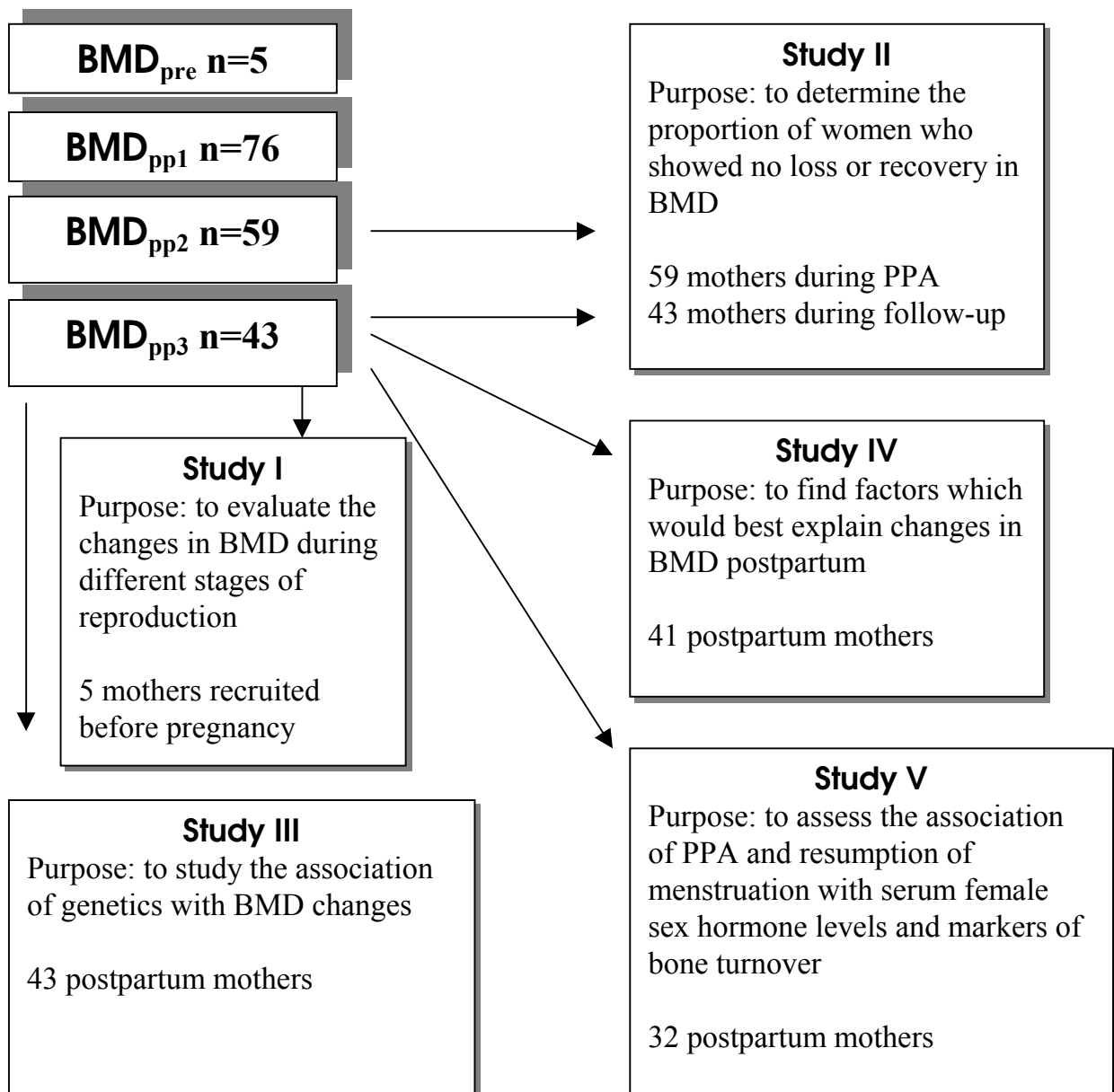
BMD_pre n=5

BMD_pp1 n=76

BMD_pp2 n=59

BMD_pp3 n=43

Study II
Purpose: to determine the proportion of women who showed no loss or recovery in BMD

59 mothers during PPA
43 mothers during follow-up

Study I
Purpose: to evaluate the changes in BMD during different stages of reproduction

5 mothers recruited before pregnancy

Study IV
Purpose: to find factors which would best explain changes in BMD postpartum

41 postpartum mothers

Study III
Purpose: to study the association of genetics with BMD changes

43 postpartum mothers

Study V
Purpose: to assess the association of PPA and resumption of menstruation with serum female sex hormone levels and markers of bone turnover

32 postpartum mothers

FIGURE 1. Scheme of the subjects in studies I-V. The BMD measurements were performed before pregnancy (BMD_{pre}), after delivery (BMD_{pp1}), after resumption of menses (BMD_{pp2}) and one year thereafter (BMD_{pp3}).

2. Measurements

2.1 Bone densitometry

Areal BMD (g/cm^2) was measured in the lumbar spine (L2 - L4), right femoral neck and dominant distal radius by dual-energy X-ray absorptiometry (Norland XR-26, Norland Inc., Fort Atkinson, WI) according to our standard procedures (Sievänen et al. 1992, 1996). All scans were performed by the same experienced laboratory technician, whose reported in vivo precision of the BMD measurements is 0.7% for lumbar spine, 0.5% for femoral neck, and 0.8% for distal radius (Sievänen et al. 1996). According to our quality assurance protocol (Sievänen et al. 1994) no significant machine drift was observed during the entire 48-month study period.

2.2 Anthropometry, dietary calcium intake, and questionnaire

Concomitant with the DXA measurements, height and weight were measured in normal indoor clothing without shoes. The subjects also completed a questionnaire on health, breastfeeding history and physical activity, and a one-week calcium intake diary (Uusi-Rasi et al. 1994). Calcium intake were analysed with Micro-Nutrica software (Social Insurance Institution, Helsinki, Finland).

2.3 Biochemical measurements

Serum total calcium (S-Ca), phosphorus (S-Pi), albumin (S-alb), 25 hydroxyvitamin D (25OHD) and intact plasma parathyroid hormone (PTH) were determined from venous blood samples. Nonfasting samples were taken coincident with visits to the well-baby clinic between 8 am and 14.30 pm. The samples were assayed in batches. S-Ca was analysed using the endpoint colorimetric (arsenazo III) dry chemistry method with Kodak Ektachem 700 (Eastman Kodak Company, Rochester, NY, USA). The intra- and inter-assay variations for S-Ca (at 1.87 mmol/l) were 0.88% and 1.14%, respectively. S-Pi was measured by

colorimetric dry chemistry with Kodac Ektachem 700 applying the manufacturer's method. The intra- and inter-assay variations for S-Pi (at 1.08 mmol/l) were 1.06% and 0.96%, respectively. S-alb was analysed based on color reaction (Hitachi 704, 705) using an in-house method (Parviainen et al. 1985). The intra- and inter-assay variations for S-alb (at 40 g/l) were 2.20% and 1.82%, respectively. The samples for 25OHD analysis were purified by the acetonitrile-C_{18} Sep-Pak method (Turnbull et al. 1982). Thereafter the metabolites were separated by high-performance liquid chromatography (HPLC), and 25OHD was measured by a method based on binding to the competing protein (Parviainen et al. 1981). PTH was determined by two-site immunoradiometric assay using reagents supplied by Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. The intra- and inter-assay variations for PTH (at 1.27 pmol/l and 4.1 pmol/l) were 3.9% and 2.9%, respectively.

The normal reference ranges were 2.15-2.60 mmol/l for S-Ca, 0.80-1.40 mmol/l for S-Pi, 36-50 g/l for S-alb and 1.0 - 6.8 pmol/l for PTH. The reference range for 25OHD was >17.7 nmol/l for winter time and 30 - 130 nmol/l for summer.

2.4 Biochemical markers of bone turnover

All samples from each subject for bone turnover markers were analysed within a single batch to minimise analytical variation. Serum bone-specific alkaline phosphatase (BAP), serum amino-terminal propeptide of type I procollagen (PINP), and serum osteocalcin (OC) were used as markers of bone formation. BAP was analysed by enzyme immunoassay (Alkphase-B, ELISA from Metra Biosystem, Icn,. Mountain View, CA, USA). The intra- and inter-assay variations for BAP (at 15.7 U/l) were 3.3% and 3.0%, respectively. PINP was analysed with commercial RIA (Procollagen Intact PINP by Orion Diagnostica, Oulunsalo, Finland). The intra- and inter-assay variations for PINP (at 159 µg/l) were 3.2% and 5.0%, respectively. OC was analysed by the immunoradiometric assay (IRMA), which measures 1-49 human osteocalcin and human osteocalcin peptide 1-43 (ELSA-OSTEO, CIS

Bio International, Gif-sur-Yvette, France). The intra- and inter-assay variations for OC (at 15 µg/l) were 1.7% and 7.2%, respectively.

Serum type I collagen carboxy-terminal telopeptide (CTx) was used as a marker of bone resorption. CTx was analysed by enzyme immunoassay (EIA); reagents were supplied by Incstar (Incstar Corp., Minnesota, USA). The intra- and inter-assay variations for CTx (at 20 µg/l) were 9.5% and 14.2%, respectively.

2.5 Female reproductive hormones

Serum estradiol (E_2), luteinising hormone (LH) and follicle-stimulating hormone (FSH) were used to describe female hormonal status. Samples were assayed in batches. E_2 was analysed by radioimmunoassay using unreactive hormone to strip estradiol from carrier proteins using reagents from Sorin Biomedica, Saluggia, Italy. The intra- and inter-assay variations (at 0.23 nmol/l and 0.26 nmol/l) were 2.9% and 4.0%, respectively. LH and FSH were analysed by immunofluorometry with two monoclonal antibodies using the reagents AutoDELFIA^{TM} hLH Spec kit B031-101 and hFSH kit B107-101 from Wallac Ltd., Turku, Finland. The intra- and inter-assay variations for LH (at 4.2 U/l) were 1.6% and 2.0% and for FSH (at 7.9 U/l and at 7.4 U/l) 1.5% and 3.8%, respectively.

The normal ranges of hormone levels observed in these assays during the follicular phase of the menstrual cycle are 0.10 to 0.45 nmol/l for E_2, 1.6 to 9.3 U/l for LH and 3 to 11 U/l for FSH.

2.6 Genotyping

VDR genotype was determined according to a method previously described (Morrison et al. 1994, Järvinen et al. 1998). Briefly, DNA was extracted from peripheral leukocytes and a 900 base pair fragment including the polymorphic site in the untranslated region at the 3'-

end of the VDR gene was amplified by polymerase chain reaction (PCR) using the primers 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and 5'-AACCAGCGGGAAGAG GTCAAGGG-3'. Fifteen µl of PCR product was subsequently incubated with *BsmI* restriction enzyme (New England Biolabs, MA, USA) and digested to fragments of 700 and 200 bp if the restriction site was present. The presence of a restriction site at the VDR gene locus was specified as b, whereas the absence of this site was B.

ER genotype was determined using a previously described method (Kobayashi et al. 1996, Yaich et al. 1992)]. Briefly, DNA was extracted from peripheral leukocytes and a 1.3 kb fragment including a part of intron 1 and exon 2 of the ER gene was amplified by PCR using the primers 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3'; 5'TCTTTCTCT GCCACCCTGGCGCGATTATCTGA-3' (Morrison et al. 1994). Fifteen µl of PCR product was subsequently incubated with *PvuII* restriction enzyme (New England Biolabs, MA, USA). The presence of the *PvuII* restriction site at the ER gene locus was specified as p, whereas the absence of this site was P.

3. Study design

Subsequent to prepregnancy DXA (BMD_{pre}) measurements, the five of six women became pregnant within 27 - 121 days. The DXA measurement at partus (BMD_{pp1}) for the basic group of 76 mothers was made within mean 6 (SD 3, range 1-15) days after delivery. Given the good indication of the first menstrual bleeding of recovered ovulatory ovarian function (Howie et al. 1982, Diaz et al. 1992), and to ensure that menstruation had actually commenced, the DXA measurement at resumption of menses (BMD_{pp2}) was to be done during the second bleeding postpartum. In fact, this was done in 47 subjects, and during the third bleeding in 10 subjects. One woman was measured during the fourth bleeding, and one had data missing on menstruation; both were excluded from further analyses (studies IV, V). The purpose of this schedule of DXA measurements was to ensure that the women were not

pregnant at the time of BMD measurement. Thus, the DXA investigation was to be carried out at BMD_{pre}, BMD_{pp2}, and BMD_{pp3} within a week after commencement of menstrual bleeding. The mean duration of the follow-up period was 12.5 (1.6) months after resumed menstruation in women who completed the entire study.

Venous blood samples were taken mean 2.0 (SD 2.1) days after delivery, 3 months postpartum concomitant with DXA measurement at resumption of menses and one year thereafter. For genotyping, venous blood samples were collected at the end of the study. All samples were frozen and stored at -70° C before assay.

The study design is presented schematically in Figure 2.
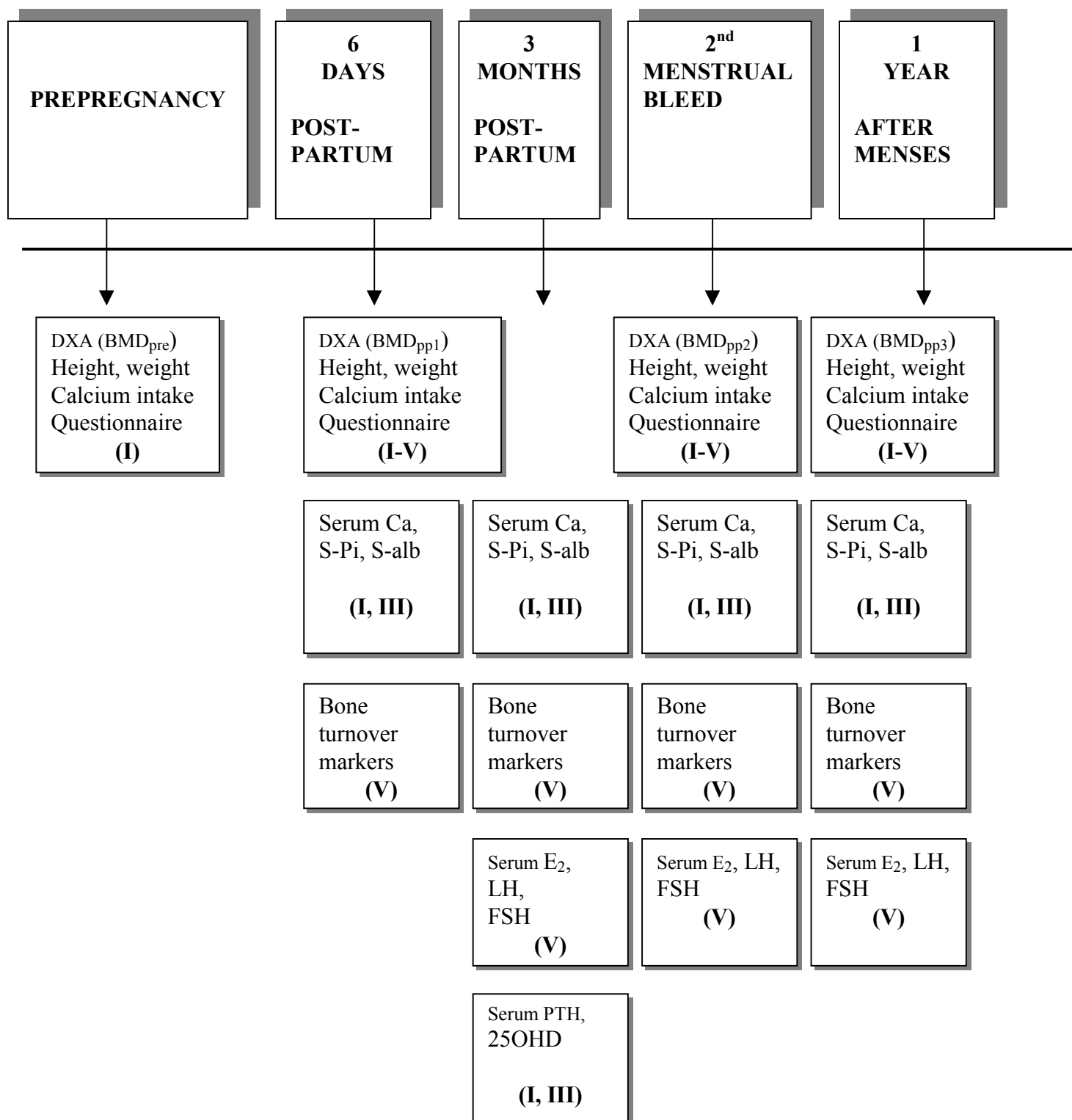
FIGURE 2. According to study protocol, measurements were to be performed before pregnancy, immediately after delivery (6 days postpartum), 3 months postpartum, one month after resumption of menses (during the second bleeding postpartum), and one year thereafter. The various studies are indicated by bold Roman numbers (I-V).

4. Statistical analysis

The descriptive values of variables were expressed as means and standard deviations (SD), 95% confidence intervals (95% CI), range and proportional changes. Distributions of treated variables were evaluated for normality, and because the distribution of female reproductive hormonal data was not normal, logarithmic transformation was applied (study V). Correlation was assessed using the Pearson correlation coefficient (r).

Changes in BMD greater than $2\sqrt{2}$ times the short-term in vivo proportionate precision (i.e., 2.0% for lumbar spine, 1.4% for femoral neck, and 2.3% for distal radius) were considered as changes with sufficient significance at individual level. Although, whether an individual subject has changed her BMD using these criteria is very unreliable, because the precision of DXA measurements vary with the individual and by definition will be poorer for subjects with low BMD, this change criterion was applied as an indicator of "real" change in study I because of the small number of subjects. Moreover, to obtain a further perspective in this study, the reproduction-related BMD changes were compared to the variability in BMD changes in healthy premenopausal women (Heinonen et al. 1996), and to the variability in BMD in a cross-sectional sample of healthy young women (Heinonen et al. 1995). In order to eliminate false-positive changes in BMD, the "real" change criterion was also used in study II, in which the proportions of women with actual bone changes were determined. The 95% CI was calculated to show significant differences in the proportions.

In general, statistical comparisons between groups were made using analysis of variance (ANOVA) and Kruskal-Wallis analysis of variance, as appropriate. Friedman's analysis of variance was used to compare changes in categorical data during the study. Paired t-test was used to compare changes in continuous variables during different phases of the study.

The significance of change in BMD at each skeletal site was determined by comparing the BMD data at postpartum and after the resumption of menstruation by a paired t-test (study IV). Differences in the rates of bone changes in postpartum women representing different

genotypes (study III), and the association of time with bone turnover marker and hormone levels (study V) were evaluated by analysis of covariance for repeated measurements (ANCOVA).

The time of recovery (T_R) to postpregnancy level (study IV) was estimated as follows:

$$T_R = T_{PPA} + T_{FU}(BMD_{pp1} - BMD_{pp2})/(BMD_{pp3} - BMD_{pp2}),$$

where T_{PPA} is the duration of PPA, and T_{FU} the duration of follow-up after the resumption of menstruation till the end of the study. A change in BMD during T_{FU} was assumed to be linear. Obviously this assumption is not an accurate description of recovery, but it provides a reasonable approximation. If an individual $BMD_{pp2} \geq BMD_{pp1}$ (i.e., no immediate, apparent bone loss took place postpartum), her T_R took on a value of 0. Be it noted that T_R was not estimated if $BMD_{pp1} > BMD_{pp2} \geq BMD_{pp3}$ (i.e., there was no trend to apparent recovery in BMD). There was one such case for lumbar spine BMD and five for femoral neck BMD. These 5 women whose T_R could not be estimated were not different from other mothers in terms of age, anthropometry, duration of lactation and PPA and life style habits. The rate of recovery after resumption of menses was also calculated as a change % divided by the duration of follow-up (in months).

Given the relatively small size of the study sample and the multiple and high correlations between independent variables, all possible subsets regression analysis based on Mallows' C_p criterion was used as our primary statistical method (Hocking 1972) to expose factors possibly contributing to change in BMD during PPA, T_R (study IV), rate of recovery during follow-up, or bone turnover immediately after parturition, 3 months postpartum, and after PPA (study V). This multiple regression analysis seeks the best combination of independent predictors out of all candidate variables served for the model.

When the absolute change in BMD during PPA was used as a dependent variable (study IV), the independent variables in the regression analysis were the duration of PPA (in months), duration of unsupplemented lactation (in months), status of present lactation at BMD_{pp2}

(lactating or not at the time of BMD_{pp2} measurement), duration of previous lactation (in months), relative weight change during PPA (in %), age (in years) and parity (in numbers).

When T_R was used as a dependent variable (study IV), duration of PPA (in months), duration of unsupplemented lactation (in months), total duration of lactation (in months), duration of previous lactation (in months), relative weight change during the study (in %), age (years) and parity (in numbers) were used as independent variables.

To expose factors underlying the rate of recovery after resumption of menses, the duration of PPA (in months), duration of unsupplemented lactation (in months), duration of total lactation, duration of previous lactation (in months), relative weight change during the study (in %), age (in years) and parity (in numbers) were used as independent variables.

When the relative marker level at a given time point in relation to baseline value (in %) was used as a dependent variable (study V), the following variables were chosen as potential independent variables at each time point. After parturition, age (in years), maternal weight after parturition (in kg), duration of pregnancy (in weeks), infant birth weight (in g), time postpartum (in days), parity (in number of children), previous lactation history (in months) were used. At 3 months postpartum, age (in years), weight (in kg), parity (in number of children), previous lactation history (in months), time postpartum (in days), menstrual status at 3 months (menstruating = 0 / amenorrheic = 1), lactation status at 3 months (not lactating = 0 / still lactating = 1), and natural logarithm of E_2 level were used. After PPA, age (in years), weight (in kg), parity (in number of children), previous lactation history (in months), duration of PPA, duration of exclusive lactation, lactation status at resumption of menses (not lactating = 0 / still lactating = 1), time since cessation of breast-feeding and logarithm of E_2 level were used.

All analyses were performed with Statistica/Win (StatSoft Inc., Tulsa, OK) on PC, and BMDP Statistical Software (Version 1993; Los Angeles, CA) on a SUN/UNIX mainframe.

RESULTS

1. Changes in clinical characteristics

The mean duration of PPA in the women who adhered to the study protocol until BMD_{2pp} (n=59) was 5.6 (SD 2.7; range 0.9 to 11.7) months. The durations of unsupplemented lactation and total lactation were 3.0 (1.3; 0.1 to 7) months and 7.8 (4.2; 0.2 to 22) months, respectively. The duration of amenorrhea correlated moderately with total and unsupplemented lactation (r=0.45, both, p<0.001). Mother's age did not correlate with these variables.

While still lactating 37 women resumed menstruation, 6 of them during the period of unsupplemented lactation. The remaining 22 resumed menstruation at a mean 1.1 (range 0.2 to 3.0) months after discontinuing lactation. A third of the mothers (21 out of 59) breastfed 9 or more months. At the time of BMD_{2pp}, 37 (63%) women had weaned. At the time of BMD_{3pp}, one mother (out of 43) had not yet weaned.

Body weight increased systematically during pregnancy (n=5) and was 7.4 (SD 1.9) kg higher after delivery as compared to prepregnancy weight. During PPA, the mean decrease in body weight in the same subjects was –6.3 (2.3) kg on average (study I), which was in accordance with significant weight loss –4.4 (3.6, p<0.001) kg seen in the larger sample (n=59). In those who completed the entire study (n=43), weight remained virtually unchanged thereafter, being at BMD_{2pp} 64.9 (11.1) kg and at BMD_{3pp} 64.8 (11.6) kg. Weight changes were similar in all VDR and ER subgroups (study III).

Mean dietary intakes of calcium well met the moderate dietary allowance for the normal healthy population (1000 mg calcium/day) during the entire study period. The intakes were significantly higher immediately after delivery than at BMD_{2pp}, i.e. 1150 (SD 550) mg/day, n=51 due to missing data, and at BMD_{3pp}, i.e. 1100 (410) mg/day, n=41. This pattern was also seen in all genotypes.

Seven women were using oral contraception at BMD_{2pp}, and ten at BMD_{3pp} (missing data on 6 and 2 women, respectively). As regards exercise, smoking, caffeine and alcohol consumption habits during the study, no significant alterations in these habits occurred according to Friedman's ANOVA. None of the mothers was engaged in athletic sports or extreme loading at any point in this study.

The mean levels of S-Ca, S-Pi and S-alb were within normal reference values during the entire study period (Table 3). They were, however, at their lowest at parturition. In fact, at that time point 21 (28%) women remained slightly below the normal reference limit for S-Ca (range 1.55 to 2.45 mmol/l). At three months postpartum the mean levels of S-Ca, S-Pi and S-alb were elevated as compared to parturition or to resumption of menses, but in line with the normal range. Greater fluctuation in S-alb levels was detected in the genotype bb group as compared to the other VDR groups (p=0.04), and in S-Pi levels in the genotype PP group as compared to the other ER groups (p=0.04). Otherwise, the analysis of covariance showed no significant interaction between genotypes for S-Ca, S-Pi, 25OHD nor PTH (study III).

TABLE 3. The descriptive biochemical and hormonal data, mean (SD), during the study.

	Partus (n=76)		3 months postpartum (n varies due missing data)		Resumption of menses (n=59)		One year after resumption of menses (n=43)	
S-Ca (mmol/l)	2.19	(0.12)	2.39[a]	(0.07)	2.31	(0.10)	2.43[a]	(0.19)
S-Pi (mmol/l)	1.21	(0.56)	1.31[a]	(0.13)	1.24	(0.14)	1.22[a]	(0.18)
S-alb g/l (mmol/l)	31.2	(4.3)	49.1[b]	(4.5)	45.3	(3.3)	43.0[a]	(3.7)
PTH (mmol/l)			3.0[a]	(1.6)				
25OHD (mmol/l)			41.3[a]	(13.1)				
S-E$_2$ (U/l)			0.09[b]	(0.02)	0.12	(0.13)	0.12[d]	(0.11)
S-LH (U/l)			6.5[b]	(7.4)	4.4[c]	(2.7)	4.3[a]	(2.3)
S-FSH (U/l)			8.7[b]	(13.1)	5.5[c]	(2.1)	6.8[a]	(4.3)

Number of subjects a=41, b=37, c=54 and d=42

2. Bone mineral density

2.1 Bone mineral changes during pregnancy

Since all subjects studied before pregnancy became pregnant within four months after the first DXA measurement, and the child of the only mother with a previous parturition was already two-years old, the baseline BMD data apparently well describe their prepregnancy skeletal status. During pregnancy there was a trend to bone loss in the lumbar spine and distal radius, but less clearly in the femoral neck (study I). Thus, as regards BMD postpartum, the skeletons of women after delivery are apparently in a fluid state (Figure 3).

2.2 Bone mineral changes during PPA

The PPA period seemed to result in systematic bone loss in the femoral neck, whereas the lumbar spine and distal radius showed diffuse responses at the individual level (study I). This was in line with the proportions of subjects with actual BMD changes in the large sample (n=59). The prevalence of significant, individual bone losses during PPA was higher at the femoral neck site [81% (95% CI 69% to 90%)] than the lumbar spine [49% (36% to 63%)] or distal radius [37% (25% to 51%)] (study II).

During PPA (n=59), the mean BMD loss was significant at all sites (Figure 3), the losses being –1.9% (95% CI –3.0% to –0.9%, p<0.001) in lumbar spine, –3.4% (–4.1% to –2.7%, p<0.0001) in femoral neck, and –1.3% (–2.2% to –0.4%, p<0.01) in distal radius. No significant VDR or ER genotype-related differences were observed between the BMD loss at any site (study III). When comparing the lactating women against those no longer lactating at BMD_{2pp}, the bone loss was statistically greater in the lactating women in the lumbar spine [–3.5% vs. –1.0%, p=0.02] and distal radius [–2.8% vs. –0.5%, p=0.01], but not at the femoral neck site, where the losses were similar [–3.8% vs. –3.2%, p=0.6], even after adjustment for weight.

Lumbar spine

Femoral neck

Distal radius

Measurement
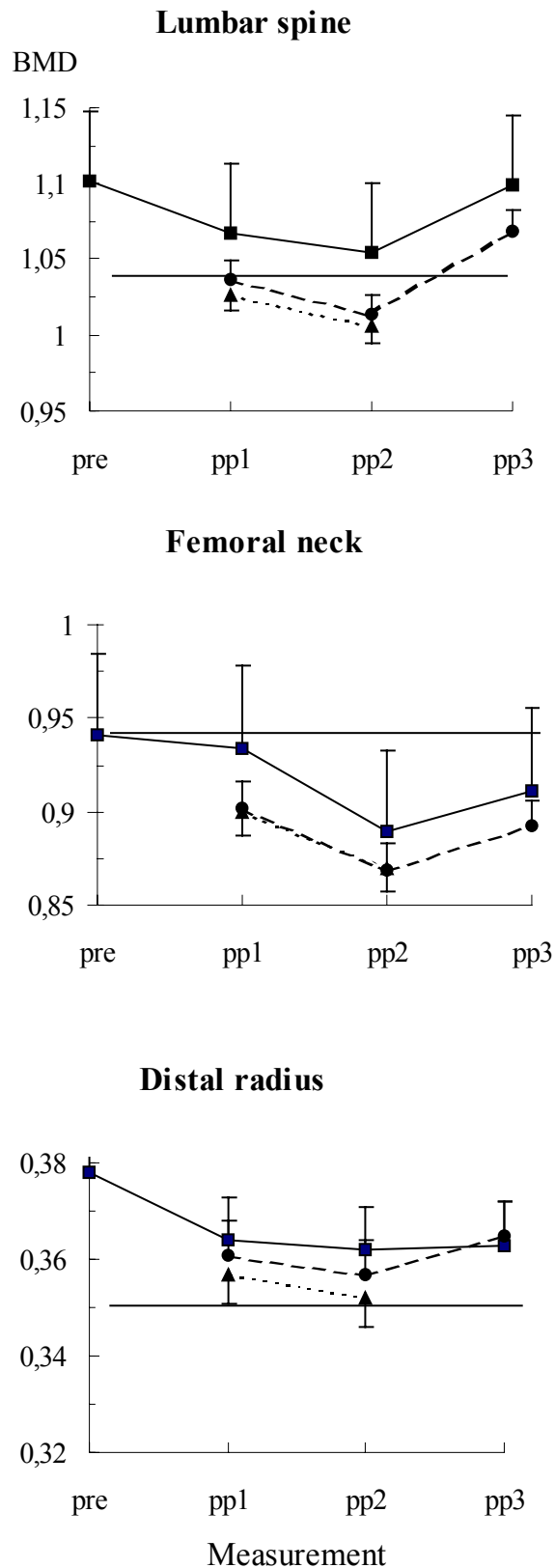
FIGURE 3. Mean absolute changes in BMD (±SEM) in lumbar spine, femoral neck and distal radius during pregnancy, PPA and one-year follow-up (■ n=5), during PPA (▲ n=59) and during PPA and one-year follow-up (● n=43). The solid line denotes the mean BMD for healthy 20 to 40 –years old females (adapted from the study by Haapasalo et al. 1996).

In study IV, different lactational variables and the duration of PPA explained significantly the site-specific changes in BMD during PPA (Table 4). Interestingly, a short PPA at the lumbar spine site but a long PPA in the femoral neck were associated with a large bone loss during PPA. A long duration of unsupplemented lactation at both sites was associated with a large bone loss, whereas a long previous lactation history or higher parity tended to protect against bone loss.

TABLE 4. Significant site-specific predictors of BMD loss during postpartum amenorrhea (n=41).

Skeletal site	Adjusted R^2	Variable	Regression coefficient	Standard error	Contribution to R^2
Lumbar spine	0.21	PPA	+0.006	0.002	0.14
		Unsupplemented lactation	−0.012	0.005	0.10
		Parity	+0.014	0.007	0.08
		Age	−0.003	0.002	0.07
Femoral neck	0.27	Previous lactation	+0.002	0.0005	0.23
		Unsupplemented lactation	−0.005	0.003	0.06
		PPA	−0.002	0.001	0.05

A positive sign of the regression coefficient indicates a smaller bone loss with a greater value of the given independent variable. A negative sign indicates a greater bone loss with a greater value of the given independent variable.

2.3 Bone mineral changes after resumption of menstruation

After resumption of menses (n=43), a trend towards recovery in BMD was observed (Figure 3). Compared to postpregnancy levels an "overcorrection" in the lumbar spine [3.3% (95% CI 2.1% to 4.5%, p<0.0001)] and the distal radius [1.1% (0.3% to 1.9%, p<0.01)] was seen at the end of the study, in contrast to only a partial recovery in the femoral neck [−1.0% (−1.8% to −0.3%, p<0.01)]. The predicted average recovery times (T_R) were 11.1 (8.6 to 13.7) months and 24.4 (14.6 to 34.2) months for lumbar spine and femoral neck, respectively. The significant predictors of T_R remained in general rather weak (study IV).

The duration of PPA was not associated with T_R, whereas the duration of unsupplemented lactation (lumbar spine, R^2 0.13) or the duration of total lactation (femoral neck, R^2 0.02) were weakly and positively associated with T_R. The rate of recovery after resumption of menses was 0.5% (95% CI 0.3% to 0.6%)/ month in the lumbar spine, 0.2% (0.1% to 0.3%)/ month in the femoral neck and 0.2% (0.1% to 0.3%)/ month in the distal radius. The predictors explaining these rates are presented in Table 5.

TABLE 5. Predictors explaining the rate of recovery after resumption of menstruation (n=41).

Skeletal site	Adjusted R^2	Variable	Regression coefficient	Standard error	Contribution to R^2
Lumbar spine	0.24	PPA	−0.055	0.020	0.14
		Previous lactation	−0.014	0.007	0.07
		Total lactation	+0.027	0.015	0.06
Femoral neck	0.06	PPA	+0.024	0.013	0.08
Distal radius	0.10	Previous lactation	−0.007	0.007	0.03

Besides the divergence in recovery rates, also the recovery patterns were diffuse at the individual level (studies I, II). At the end of the study (study II), in only 2 women was lumbar BMD not fully recovered to postpregnancy level, while 25 [58% (95% CI 42% to 73%)] showed even higher lumbar BMD at the end than at postpartum. The mean bone gain in these subjects was 6.0 (SD 0.6)% as compared to postpartum BMD. In contrast, at the femoral neck, 18 [42% (27% to 58%)] did not reach the postpartum BMD, the mean bone loss in these subjects being −3.2 (0.4)%. The results at the distal radius were less distinct. Despite breastfeeding, all those 15 women who continued lactation after BMD_{pp2} measurement increased their BMD in the lumbar spine, 10 in the femoral neck, and 8 in the distal radius during the subsequent follow-up, and only 3 lost further bone in the femoral neck (study II). At the end of the study, BMD data on mothers who did not breastfeed during the follow-up period compared to those who breastfed did not differ from each other (study IV). No significant VDR or ER genotype-related differences were observed between BMD recovery at any site (study III).

3. Postpartum bone turnover

Bone turnover markers were in an elevated state immediately after parturition (study V). All marker levels changed significantly (p=0.0036) postpartum and evinced different patterns with time (p=0.0001). Indices of bone formation, PINP and OC increased up to 3 months while BAP showed a decreasing trend (Figure 4). After 3 months, the markers of bone formation started to decrease systematically. In contrast to bone formation markers, the bone resorption marker CTx started to decrease after parturition. Of note was that, the bone formation markers and the resorption marker did not correlate at resumption of menstruation.
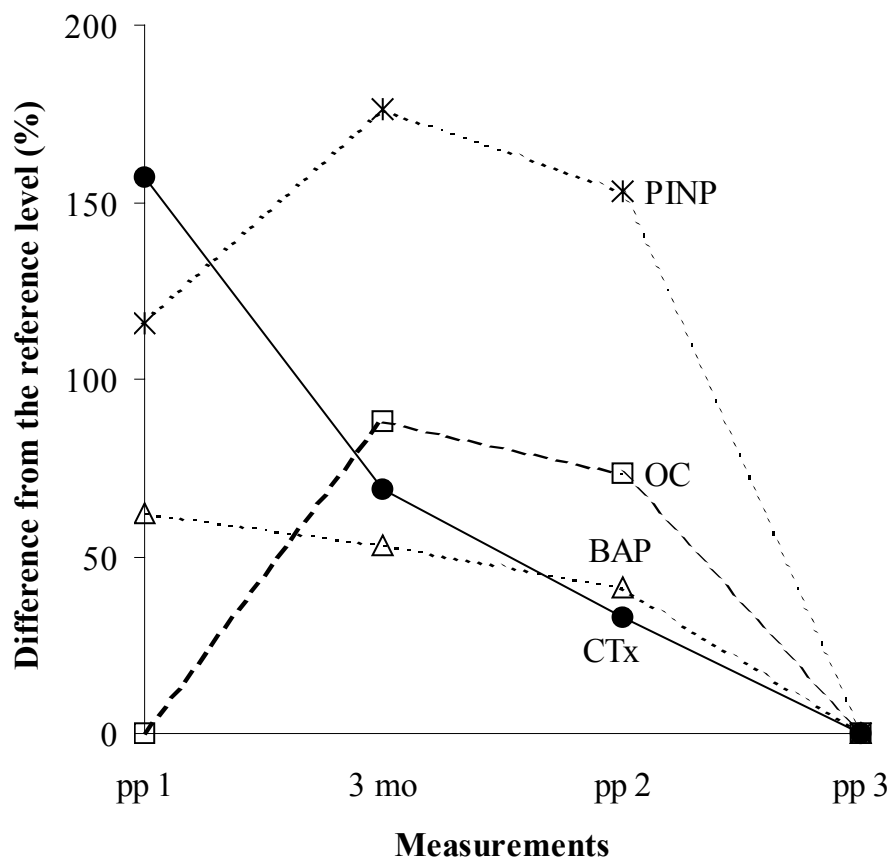
FIGURE 4. Dynamic pattern of bone turnover markers from partus (pp1) to 3 months postpartum (3 mo), after resumption of menses (pp2) and one year after resumption of menses (pp3). The mean times between pp1 and both pp2 and pp3 are 7.1 (range 2.7 to 12.8) months and 19.3 (range 14.3 to 26.0) months, respectively. The values at the end of the study were used as reference level.

At 3 months after parturition (Table 6), the E_2 level was negatively associated with all bone formation markers; the lower the E_2 level, the higher the formation marker levels. The lactation status was weakly and positively associated with increased PINP levels. In fact, lactation seemed to indicate in general a high bone turnover state. If the mother was lactating and still amenorrheic at 3 months postpartum, her bone resorption marker level was also likely to be higher than in mothers who were menstruating and not lactating. After the resumption of menstruation (Table 7), the duration of unsupplemented lactation was positively associated with high levels of bone formation markers, as was the duration of postpartum amenorrhea; the longer the unsupplemented lactation and PPA, the more increased the indices of bone formation.

In addition, at 3 months after parturition, high parity was associated with lower levels of formation markers, whereas high age as such indicated increased formation. After the resumption of menstruation, a history of previous lactation or parity indicated a low bone turnover state in general, while age as such was positively associated with high bone formation. It is significant that these same variables which were predictors of bone turnover were also predictors of changes in BMD.

TABLE 6. Significant predictors of bone turnover at 3 months postpartum (n=32).

Bone marker	Adjusted R^2	Variable	Regression coefficient	Standard error	Contribution to R^2
BAP	NS	–	–	–	–
PINP	0.25	Parity	–0.530	0.212	0.15
		E_2	–0.549	0.239	0.13
		Age	+0.066	0.041	0.06
		Lactation status	+0.817	0.544	0.05
OC	0.26	E_2	–0.357	0.122	0.20
		Age	+0.055	0.021	0.16
		Parity	–0.254	0.109	0.13
CTx	0.29	Menstrual status	+0.484	0.184	0.16
		Lactation status	+0.683	0.267	0.15
		Weight	+0.018	0.008	0.12

Note: A positive sign of the regression coefficient in independent variables lactation and mentrual status denote continued lactation or amenorrhea with a higher level of the marker. NS not so significant

TABLE 7. Significant predictors of bone turnover after resumption of menstruation (n=32).

Bone marker	Adjusted R^2	Variable	Regression coefficient	Standard error	Contribution to R^2
BAP	0.22	Previous lactation	–0.038	0.015	0.16
		E_2	–0.268	0.126	0.11
		Exclusive lactation	+0.184	0.091	0.10
		Age	+0.044	0.022	0.10
		PPA	+0.051	0.032	0.06
PINP	0.14	Parity	–0.366	0.214	0.08
		Exclusive lactation	+0.297	0.171	0.08
OC	0.20	Previous lactation	–0.029	0.013	0.13
		Exclusive lactation	+0.160	0.074	0.12
		PPA	+0.051	0.027	0.09
		Age	+0.034	0.019	0.09
CTx	NS	–	–	–	–

Note: Previous lactation, exclusive lactation, and PPA denote the duration of the given variable.
NS not so significant

DISCUSSION

Changes in BMD during successive stages of reproduction (i.e., during pregnancy, PPA and one-year follow-up) at functionally and compositionally different skeletal sites (lumbar spine, femoral neck and distal radius) have not previously been reported. In contrast to fixed measurement schedules in the literature, the second postpartum BMD measurements in the present study were carried out on a physiological basis after the resumption of menstruation. As postmenopausal bone loss is considered to be a consequence of hypoestrogenenemia (Riggs et al. 1998, Slemenda et al. 1987) and fertile women are hypoestrogenic until the resumption of menstruation (Howie et al. 1982, Diaz et al. 1992), we addressed this question by measuring BMD soon after the resumption of menses, at the time when the skeletal status was anticipated to represent the "worst case" situation due to the combined effects of amenorrhea and calcium loss into breast milk with unsupplemented lactation during the first months postpartum. This approach introduces a new viewpoint to postpartum studies. Such physiology-based scheduling of bone mass measurements may allow a more appropriate analysis of the effects of resumed hormonal activity (as judged from resumed menstruation) on bone than the conventional mode based on fixed time points. The strength of the present study was the longitudinal follow-up of a cohort of postpartum women at the price, however, of an obviously small number of subjects who completed the entire study protocol. The lack of concurrent control data from nonlactating, healthy women should likewise be noted. Also, the possibility cannot be ruled out that the changing body composition, thickness and/or tissue distribution during different phases of this study affected DXA-measured BMD values and confounded the results (Bolotin 1998, Bolotin et al. in press). As regards the changes in the laboratory data, practical reasons dictated that the serum samples were not drawn after overnight fasting and exactly at the same time of the day. Consequently, the variation in the laboratory data may well have increased, but this situation hardly had caused any systematic error in trends or magnitudes in given data.

1. Pregnancy-related changes in bone mineral

The heterogeneity of individual BMD changes in response to pregnancy and lactation in the study I are some of the most interesting questions still to be answered. However, during pregnancy there was some tendency towards systematic bone loss in the lumbar spine (about −3% on average), whereas particularly the femoral neck showed no such clear trend (study I). Given the small sample size of five mothers, and the consequently limited statistical power, this conclusion is suggestive only and should be interpreted with caution. Nevertheless, this finding is in line with those presented in the literature (Drinkwater and Chesnut 1991, Sowers et al. 1991, Black et al. 2000, Naylor et al. 2000), though not consistently (Ritchie et al. 1998). Previous studies of urine bone resorption markers (Cross et al. 1995a, Ritchie et al. 1998, Black et al. 2000, Naylor et al. 2000) and serum bone formation markers (Black et al. 2000, Naylor et al. 2000) suggest that bone turnover increases during pregnancy, showing a dissociation between bone resorption (i.e. increasing trend) and formation (i.e. decreasing trend) during the first half of pregnancy (Black et al. 2000, Naylor et al. 2000). These observations also speak for skeletal changes observed during pregnancy. The immediate postpartum levels of bone turnover in the present study, indicating a high bone turnover state at the time of parturition, are in perfect congruence with these studies, and as regards the BMD postpartum, this high turnover pinpoints the fact that the skeletons of women are in a fluid state after delivery. It is also worth noting that some individuals may well show large, systematic bone losses during pregnancy despite substantial weight gain (study I).

2. Postpartum changes in bone mineral

In the present cohort of healthy postpartum women, a significant mean (depending on site some 2% to 3%) bone loss took place in the weight-bearing skeleton during PPA, and even a slight "overcorrection" compared to postpregnancy levels was observed in the lumbar spine in contrast to only a partial recovery in the femoral neck. These site-specific patterns of

postpartum bone loss and recovery may arise from the different bone composition of the given skeletal sites, of which the lumbar spine has a substantially higher trabecular to cortical bone ratio and thus more efficient metabolism than the femoral neck (Baron 1993, Einhorn 1996). Net gains in lumbar BMD after lactation relative to postpartum measurements have also been documented in other studies (Lopez et al. 1996, Laskey et al. 1999, Polatti et al. 1999). Lower BMD at the femoral neck has been reported (Sowers et al. 1993, Kolthoff et al. 1998, Laskey et al. 1999), but not in all studies (Lopez et al. 1996). Also, only partial recovery in femoral neck BMD involving about a half of postpartum women indicates that the postpartum period may predispose the proximal femur to a risk of permanent, although relatively small bone loss (study II). However, femoral neck may need more time to recover, and in this respect, the duration of our study may have been too short. Age-related mineral losses cannot be explored either, as no controls included in the study.

As regards the genetic impact, the bone changes were so similar in terms of both magnitude and direction irrespective of the VDR or ER genotype that, despite the small sample size, it is reasonable to conclude that the vitamin D receptor or the estrogen receptor polymorphisms may not play a strong, if any, role in postpartum amenorrhea- or lactation-related BMD changes in postpartum women (study III). Recently, also Laskey and colleagues (1998) reported that decreases in BMD after 3 months lactation were not related to VDR genotype.

In a normal hormonal milieu, the most important influence on bone mass comes evidently from mechanical loading: i.e., due to the combined effects of muscle activity and weight-bearing (Lanyon 1996). Thus, a substantial reduction in customary daily loading may result in bone loss. However, neither the previous studies of postpartum women (Caird et al. 1994, Kolthoff et al. 1998, Laskey et al. 1998, Hopkinson et al. 2000) nor the present study suggest that particularly the mothers' rapid weight loss after delivery and during PPA (some 4 kg) was associated with simultaneous or subsequent bone loss (studies III, IV). Moreover, during pregnancy when more than 10% gains in body weight occurred and the subjects maintained their living habits virtually unchanged, there seemed to be no clear relation

between changes in body weight and BMD. After the resumption of menstruation, recovery in BMD in lumbar spine and femoral neck was not related to body weight changes: in fact, the mean body weight was quite stable during the whole one-year follow-up period (i.e., 64.9 kg at resumption of menses and 64.8 kg at the end of the study). It is possible that the strong influence of coincident hormonal changes postpartum covered the potential effect of weight reduction on bone, or simply the influence of weight change in conjunction with virtually maintained physical activity level remained negligible as regards the bone loading.

3. Postpartum amenorrhea, lactation and bone turnover

The duration of lactational amenorrhea is known to be highly variable among different communities (Howie et al. 1982, Caird et al. 1994, Kalkwarf and Specker 1995) and even among mothers with comparable nursing practices (Diaz et al. 1991). Moreover, the duration of breastfeeding or the suckling pattern only partially explain the duration of lactational amenorrhea (Diaz 1989, McNeilly 1993, Vestermark et al. 1994). The correlation between duration of lactation and PPA observed in our sample was relatively weak (r=0.45), especially when compared to published information in which the correlation is usually higher, r>0.60 (Rosner and Schuman 1990, Vestermark et al. 1994). Lactation for more than 9 months has been reported not to prolong the postpartum amenorrhea (Vestermark et al. 1994). In our sample a third of the mothers breastfed 9 or more months. So this fact may have played some role behind the observed weak correlation. The correlation will be also decreased both by women who resume menstruation very early while still in full lactation and then continue to lactate for many months, and by women who lactate for several years after resumption of menstruation. In our sample, six mothers resumed menstruation during unsupplemented breastfeeding continuing lactation for some three to six months. Only one of these early menstruating mothers continued breastfeeding at one year. Women who resumed menses after cessation of lactation mostly resumed menses quite soon after weaning, except four women who resumed menstruation some two or more months after weaning. The weak correlation may also indicate some inherent feature in Finnish women.

After the first few days after parturition, it was obvious that bone turnover becomes increasingly more closely associated with menstrual and lactational factors (study V), which were also related to changes in BMD (study IV). Surprisingly, a short PPA period was associated with greater bone loss in the lumbar spine, whereas previous studies of postpartum women have shown that lactating women whose menses resume earlier also sustain smaller bone loss (Kalkwarf and Specker 1995, Sowers et al. 1996, Kolthoff et al. 1998). It is possible that in this study the actual nadir in lumbar BMD may have occurred between the BMD_{pp1} and BMD_{pp2} measurements in many mothers with a long PPA, but coincided with virtually the BMD_{pp2} measurement in women with a short PPA. If so, the inverse correlation observed in this study can be explained. This point derives further support from several studies (Sowers et al. 1993, Caird et al. 1994, Kalkwarf and Specker 1995, Kolthoff et al. 1998, Hopkinson et al. 2000), which have employed fixed measurement intervals (3 or 6 months vs. the PPA-based interval employed in the present study). In those studies the percentage change in lumbar BMD has been double compared to the present findings. Moreover, the dissociation of bone resorption (i.e. decreasing trend) and formation (i.e. increasing trend) after parturition observed here would indicate that the bone loss most likely occurs within the very first few months postpartum. In some previous studies the bone loss has indeed apparently taken place during the first 3 months postpartum (Kalkwarf and Specker 1995, Kolthoff et al. 1998, Hopkinson et al. 2000).

The duration of unsupplemented lactation accounted significantly for bone loss during PPA. This relationship is obvious considering the ample breastfeeding, which also effectively suppresses the hypothalamus-pituitary-ovarian axis (Diaz et al. 1992). The actual effect of lactation on BMD, however, is a complex issue to address since the precise total amounts and composition of milk produced during the breastfeeding period are very labor-intensive to collect (Laskey et al. 1998). Given the new approach to the study design (i.e., a physiology-based schedule of BMD measurements), and two different phenomena studied (i.e., bone loss and recovery), the interpretation of multifactorial and interrelated predictors explaining the corresponding changes in BMD is extremely involved. While at 3 months postpartum active lactation was related more to elevated bone resorption marker level than

to formation marker levels, the relationship was the opposite after the resumption of menstruation; mothers who had breastfed exclusively for a long period seemed to evince more elevated levels of bone formation. Mothers who had a long PPA were inclined to have more elevated levels of bone formation markers at resumption of menses. In other words, a long PPA and a long duration of unsupplemented lactation were both associated similarly but independently with elevated bone formation markers after PPA. As regards the BMD recovery to postpregnancy level, the predictors for T_R (study IV) and the rate of recovery (Table 5) indicate that bone recovery is modulated by lactation habits. However, as regards the magnitude and time of recovery, it must be remembered that the BMD change was assumed to be linear and the postpartum BMD may not necessarily represent the true baseline level before pregnancy. In general, the present findings were consistent with the observations of others. First, bone turnover is shown to be higher in breastfeeding than in bottle-feeding mothers (Sowers et al. 1993, Lopez et al. 1996, Kent et al. 1990, Krebs et al. 1997, Affinito et al. 1996, Dobnig et al. 1995, Zinaman et al. 1990, Caird et al. 1994, Yamaga et al. 1996). Second, the duration of lactation is a significant determinant of bone turnover (Yamaga et al. 1996, Sowers et al. 1995). Third, a high rate of bone formation is observed in women who have been lactating for a long period (Sowers et al. 1995). Fourth, a high bone formation in late lactation and postweaning is also well established (Cross et al. 1995a, Ritchie et al. 1998, Lopez et al. 1996, Kent et al. 1990, Sowers et al. 1995). It is thus obvious that this continued high bone formation contributes to the documented recovery in BMD after weaning (Cross et al. 1995a, Ritchie et al. 1998, Kent et al. 1990, Sowers et al. 1995).

Parity and accompanying previous lactation history were associated with lower bone turnover markers during the whole PPA period. In other words, previously nulliparous women may thus show more accelerated bone turnover and subsequent bone loss during PPA than parous women with a previous lactation history at the given age. The mechanism behind the potentially protective role of these factors against bone loss during PPA in the weight-bearing skeleton is intriguing but remains obscure. However the case may be, these factors are associated with prevention of bone loss during PPA rather than with enhanced

bone recovery. This points clearly to the apparent protective nature of previous lactation and parity against the loss in BMD during PPA. The finding is concordant with a recent prospective study (Hopkinson et al. 2000). In this respect, epidemiological data on previous lactation history or parity also indicate positive associations of these factors with bone mass (Feldblum et al. 1992, Berning et al. 1993). However, the epidemiological evidence is not fully convincing in that no effect (Kritz-Silverstein et al. 1992, Melton et al. 1993, Sinigaglia et al. 1996, Henderson et al. 2000) or even a detrimental effect (Lissner et al. 1991, Ghannam et al. 1999) has also been reported.

4. Hormones and postpartum bone mineral changes

Estrogen is considered the main hormonal regulator of bone metabolism in women (Turner et al. 1994, Väänänen and Härkönen 1996). Bone loss due to menopause (Riggs et al. 1998) or premenopausal amenorrhea of other causes (Schachter and Shoman 1994, Miller and Klibanski 1999) is believed to be a direct consequence of estrogen deficiency. The hypoestrogenic state in postpartum women, too, is postulated to lead to accelerated bone turnover (Hillman et al. 1981, Zinaman et al. 1990, Scharla et al. 1990) and subsequent bone loss (Sowers et al. 1996, Honda et al. 1998). The influence of estrogen on bone turnover is obvious given its direct action on bone cells via the presence of ER in human osteoblasts (Eriksen et al. 1988, Komm et al. 1988) and osteoclasts (Pensler et al. 1990). As the duration and the severity of hypoestrogenemia are reported to vary greatly among postpartum women (Shaaban et al. 1987), we anticipated that ER polymorphism might result in inter-individual variation in bone homeostasis, but in fact encountered no such trend (study III). Previously, ER polymorphism has been suggested to modulate bone metabolism (Mizunuma et al. 1997), but no significant inter-genotype differences in respect of the changes in BMD were observed in the study in question.

It is obvious that estrogen levels are suppressed in lactating women (Caird et al. 1994, Dobnig et al. 1995, Sowers et al. 1996, Lopez et al. 1996, Krebs et al. 1997, Sowers et al. 1998) and that the ensuing hypoestrogenemia predominates at least during the first 3 months postpartum (Shaaban et al. 1987, Burger et al. 1994, Dobnig et al. 1995), when this condition starts declining and becomes negligible within 5 to 6 months of lactation (Shaaban et al. 1987, Dobnig et al. 1995). In our study (study V), serum estradiol corresponded to a low follicular phase level at 3 months postpartum, and at the same time the low estrogen level was associated with less markedly increased levels of bone formation markers, while the amenorrheic state in general indicated a higher bone resorption level.

Elevations in serum calcium have been reported to accompany increased bone resorption during estrogen deficiency (Scharla et al. 1990). In agreement with observations elsewhere (Greer et al. 1982, Cross et al. 1995a, Dobnig et al. 1995, Prentice et al. 1998) we found low total calcium levels at partus which subsequently increased. Unchanged calcium concentrations have also been reported (Kent et al. 1990, Cross et al. 1995b, Caird et al. 1994, Krebs et al. 1997, Specker et al. 1991).

Soon after parturition a rapid decline in the level of bone resorption marker was observed, while bone formation markers continued increasing and eventually coincided with the resorption level within the first few months postpartum. This dissociation between bone resorption (i.e. decreasing trend) and formation (i.e. increasing trend) after parturition is dissimilar to that observed after the menopause, which is characterised by pronounced acceleration in both bone resorption and bone formation (Garnero et al. 1996). Consequently, estrogen may have a specific influence on bone turnover during the first few months postpartum, while thereafter its role as the predominant factor behind the postpartum bone losses may vanish in late PPA. A possible explanation for the impact of estrogen at the beginning of postpartum may pertain to the fact that the maternal skeleton is first accustomed to continuously elevating estrogen levels during pregnancy, this being followed by the intrinsic collapse in estrogen levels subsequent to delivery. This drastic change in estrogen levels may then transiently disturb the skeletal homeostasis and lead to specific

characteristics seen in bone turnover after parturition. Moreover, the subsequent period of lactation may further potentiate the initially high resorption level present at parturition (Black et al. 2000, Naylor et al. 2000), leading to a cumulative impact of estrogen deficiency and milk production in lactating women.

If hypoestrogenemia disappears and bone turnover balance turns from resorption to formation within a few months time from parturition, the demands of continued milk production in lactating women on extracellular calcium homeostasis may lead to substantially increased PTH secretion later in postpartum (Greer et al. 1982, Kent et al. 1990, Specker et al. 1991, Verhaeghe and Bouillon 1992, Cross et al. 1995 a,b, Affinito et al. 1996, Lopez et al. 1996). However, the evidence on classical calciotropic hormones suggests that they do not play any central role in bone metabolism accompanying human lactation (Kent et al. 1990, Specker et al. 1991, Cross et al. 1995a, Sowers et al. 1995, Lopez et al. 1996, Prentice et al. 1998, Sowers et al. 1998). Neither is the role of the vital hormone of lactation, prolactin, well established (Lopez et al. 1996, Sowers et al. 1996). There is also some evidence to suggest that parathyroid-related peptide (PTHrP) produced by the mammary gland, and possibly modulated by prolactin, may regulate maternal calcium and bone metabolism during lactation, particularly during the early weeks after parturition (Dobnig et al. 1995, Sowers et al. 1996). We did not evaluate these potentially relevant hormones, and its is thus possible that they may have accounted to some extent for the time-specific models of bone turnover observed in this study.

SUMMARY AND CONCLUSIONS

The purpose of these studies was to evaluate prospectively reproduction-related changes in BMD and associated factors during PPA with a novel physiology-related study design. BMD of the lumbar spine, femoral neck and distal radius, maternal weight and height, dietary calcium intake, questionnaires on health, breastfeeding and living habits, polymorphism of VDR and ER genes, several female sex hormones, bone turnover markers and other biochemical measurements were assessed during PPA and in a one-year follow-up period after resumption of menstruation in a cohort of healthy Finnish mothers. The baseline measurements immediately after delivery were made in 76 postpartum women aged 30.4 (SD 4.3) years. Fifty-nine mothers adhered to the study protocol until the end of PPA. The mean duration of PPA was 5.6 (2.7) months and 37% of mothers were still breastfeeding. Forty-three women completed the entire study protocol. Of these women, five were also studied before pregnancy.

The main limitation of this prospective cohort study was the relatively small sample size, and thus the following conclusions should be considered suggestive. Also, some potential predictors such as the influence of preceding pregnancy, calcium intake, use of oral contraception and substantial changes in lifestyle were not included in the analyses. Nevertheless, keeping in mind these limitations and the new approach to the study of postpartum skeletal changes, we conclude that:

1. during pregnancy there is a tendency towards systematic bone loss especially in the lumbar spine. At the time of parturition a high bone turnover state prevails. Thus, the maternal skeleton is in a fluid state after delivery;

2. during PPA a systematic and significant (depending on site some 2% to 3%) mean bone loss affects the weight-bearing skeleton, and after the resumption of menstruation bone begins to recover even despite continued lactation. The patterns of postpartum bone loss and recovery seem to be site-specific. From the clinical point of view, the magnitude of these

changes is in general relatively small, but they are greater than seen in postmenopause within such a short period and not normally observed in healthy premenopausal women;

3. some individuals may evince substantial systematic bone losses comparable to one SD in magnitude during reproduction and make only a partial recovery after resumption of menstruation. Keeping in mind the duration of the follow-up and lack of control group in this study, it is still possible that reproduction can predispose especially the proximal femur of some mothers to a risk of permanent bone loss. Recognition of these potential risk cases is undoubtedly of clinical relevance;

4. the bone turnover is increased during PPA, all bone turnover marker levels still being elevated after the resumption of menstruation. Subsequent to parturition, bone resorption apparently overcomes bone formation, but after some months bone formation becomes the predominant modality of bone turnover. This explains the well-documented bone loss during early lactation and PPA and likewise the subsequent bone recovery;

5. the dynamic pattern of bone turnover markers related to postpartum amenorrhea is dissimilar to that prevalent in menopause, which indicates multifactorial and complicated mechanisms behind the changes in bone turnover and bone mineral after parturition. Consequently, estrogen may have a specific influence on bone turnover during the first few months postpartum, but later on, the hypoestrogenemia during PPA may not be the predominant factor underlying postpartum bone losses. This would suggest that the bone loss occurs largely during the very first few months postpartum;

6. the bone turnover and BMD changes seem to be to some extent modulated by lactation habits. Especially, prolonged unsupplemented lactation is associated with greater bone loss, thus requiring more time to recover. Previous lactation history and parity, in turn, seem to provide some protection against the loss in BMD and high bone turnover during PPA;

7. vitamin D or estrogen receptor polymorphisms seem not to have a strong impact, if any, on the postpartum amenorrhea- or lactation-related BMD changes in postpartum women.

In summary, this study confirmed the presence of systematic BMD changes during different phases of reproduction. These changes appear to be site-specific and largely reversible. However, some individuals may sustain considerable, systematic bone losses, which are not completely recovered, this possibly increasing the fracture risk in these subjects in later life. Reproduction-related skeletal adaptation is a complex, multifactorial phenomenon whose time course is modulated by independent actions and interplay of several physiologic, nutritional and environmental factors, the most important being reproduction-related hormonal changes, intensity and duration of lactation and previous reproductive history.

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