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Abstract

Emerging evidence suggests that calcium and its regulating hormones, i.e. vitamin D and parathyroid hormone (PTH), affect breast cancer risk.

The associations between serum calcium levels and breast cancer risk, between serum calcium levels and known risk factors of breast cancer, and between serum calcium levels and breast cancer aggressiveness were examined within the Malmö Preventive Project, a population-based cohort comprising 10,902 women. Serum calcium, 25-hydroxyvitamin D (25OHD) and PTH levels were furthermore examined in relation to breast cancer risk in a nested case-control study comprising 764 breast cancer cases within the Malmö Diet and Cancer Study.

Serum calcium levels were positively associated with breast cancer risk in overweight/obese women. In premenopausal women, serum calcium was in one study negatively, and in one study positively, associated with breast cancer. Calcium was positively associated with breast cancer aggressiveness in overweight and/or premenopausal women. Premenopausal status and use of oral contraceptives and hormone-replacement therapy were negatively associated with serum calcium levels. BMI was significantly associated with serum calcium levels, with lean and overweight women having higher calcium levels than women with BMI between 20 and 25.

There was a weak, statistically non-significant, inverse association between 25OHD levels and breast cancer risk. There was no evidence for any relation between PTH levels and breast cancer.

It is concluded that serum calcium is positively associated with breast cancer risk and aggressiveness in overweight women. There may be a weak negative association between vitamin D and breast cancer risk, but this will have to be further examined.

Key words:
calcium; breast cancer; vitamin D; parathyroid hormone; body mass index; menopause.

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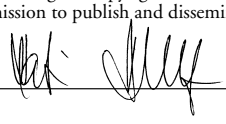
Signature  Date February 9, 2009

Till Camilla

In the long run, men hit only what they aim at.
Therefore, they had better aim at something high.
Henry David Thoreau (1817–1862)

Principal supervisor:	Jonas Manjer
Assistant supervisors:	Anne-Greth Bondeson and Lennart Bondeson
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Contents

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I Almquist M, Manjer J, Bondeson L, Bondeson AG. Serum calcium and breast cancer risk: results from a prospective cohort study of 7,847 women. *Cancer Causes Control* 2007; 18:595–602 *

II Almquist M, Bondeson AG, Bondeson L, Halthur C, Malm J, Manjer J. Reproductive history, lifestyle factors and season as determinants for serum calcium concentrations in women. *Scand J Clin Lab Invest* 2008; 68:777–85 **

III Almquist M, Anagnostaki L, Bondeson L, Bondeson AG, Borgquist S, Landberg G, Malina J, Malm J, Manjer J. Serum calcium and tumour aggressiveness in breast cancer – a prospective study of 7,847 women. Submitted.

IV Almquist M, Bondeson AG, Bondeson L, Malm J, Manjer J. Serum levels of vitamin D, PTH, calcium and breast cancer risk – a prospective nested case-control study. Submitted.

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Abbreviations

$1,25OH_2D$	1,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
CaSR	Calcium-sensing receptor
CI	Confidence interval
HHM	Humoral hypercalcemia of malignancy
HRT	Hormone replacement therapy
IQR	Interquartile range
MDCS	Malmö Diet and Cancer Study
MPP	Malmö Preventive Project
NPI	Nottingham Prognostic Index
OC	Oral contraception
OR	Odds ratio
pHPT	Primary hyperparathyroidism
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
RDA	Recommended daily allowance
RR	Relative risk
SIR	Standardised incidence ratio
TNM	Tumour, Node, Metastasis
UICC	International Union against Cancer
SCDR	Swedish Cause of Death Registry

Introduction

Breast cancer is the most common cancer in females. A growing body of literature, including epidemiologic, clinical and experimental research, suggests that calcium and its regulating hormones, i.e. vitamin D, parathyroid hormone, PTH, and PTH-related peptide, PTHrP, may affect breast cancer risk [1–11]. PTH and vitamin D regulate the production of each other, and both factors increase serum calcium levels [12].

Experimental studies have shown that 1,25-dihydroxyvitamin D (1,25OH_2D), the biologically active form of vitamin D, can inhibit cellular proliferation, induce apoptosis and inhibit angiogenesis in normal and malignant breast cells [13]. Currently, vitamin D status in humans is considered to be best estimated by measuring plasma 25-hydroxyvitamin D (25OHD) levels [14] and three prospective case-control studies have found a weak, statistically non-significant negative association between 25OHD levels and breast cancer risk [15–17]. A randomised trial found a reduction of breast cancer incidence in postmenopausal women treated with vitamin D and calcium supplements [9]. In that trial, there was also a statistically significant negative association between 25OHD levels and cancer risk.

Many epidemiologic investigations have found an increased risk of breast cancer in relation to primary hyperparathyroidism, a condition characterised by increased levels of PTH [1–3, 18]. Experimental studies also suggest that PTH has carcinogenic and tumour promoting effects [19–21].

Serum calcium *per se* might affect breast cancer incidence and aggressiveness. Calcium is an important intracellular messenger that is involved in processes related to proliferation, apoptosis and cell signalling [22]. Increasing calcium concentrations decreases proliferation and increases differentiation in breast cancer cell lines [13], which would imply a tumour protective effect. The calcium-sensing receptor, CaSR, is expressed both in normal [23] and malignant breast cells [24] and its expression seems to be correlated with skeletal metastasis [24, 25], suggesting a link between serum calcium and breast cancer aggressiveness.

Calcium metabolism might also be influenced by reproductive factors [26, 27]. It is possible to hypothesise that interactions between vitamin D, PTH and calcium and reproductive factors modify any associations between these factors and breast cancer risk.

Furthermore, several conditions may modify the relation between vitamin D, PTH, calcium and the risk of breast cancer. High age and obesity are associated with altered calcium metabolism [28, 29], high levels of PTH [30, 31], low levels of 25OHD [32], and, at least in postmenopausal women, an increased risk of breast cancer [33, 34]. It has also been suggested that pre- and postmenopausal women may have different risk factors for breast cancer [35] and that obesity may modify the association between established risk factors and breast cancer [34]. Several known risk factors of breast cancer, for example age, obesity, and use of HRT [36, 37] have been shown to specifically affect tumour biology, increasing or decreasing the risk of more aggressive tumours. Indeed, a previous study has suggested that high vitamin D intake may decrease overall breast cancer risk and that this decreased risk was especially related to aggressive breast cancers [38].

Aim of the thesis

The aim of this thesis was to study the relations between calcium, the calcium-regulating hormones, i.e. vitamin D and PTH, and breast cancer in two large cohorts: the Malmö Preventive Project (MPP), including 10,902 women altogether, with information on serum calcium levels and established risk factors of breast cancer at baseline; and the Malmö Diet and Cancer Study

(MDCS), comprising 17,035 women, with serum samples available for analysis.

Specifically, it was hypothesised that:

- Serum calcium levels are positively associated with breast cancer risk
- Serum calcium levels are correlated to female reproductive factors, age and anthropometric factors
- Serum calcium levels are associated with breast cancer aggressiveness, as determined by the risks of biologically different breast cancer subgroups
- Vitamin D, as measured by 25OHD levels, is negatively, and PTH levels are positively, associated with breast cancer risk

Breast cancer

Breast cancer is by far the most common cancer in women in the Western world, comprising around 30% of all cancers in women [39], afflicting around 7000 women yearly in Sweden [40]. There are annually 1.15 million new breast cancer cases worldwide. The life-time risk of breast cancer in women in the industrialised world is estimated to be around 11% [33].

Even though screening, diagnosis and treatment have improved so that 5-year survival in the U.S. is now about 89%, breast cancer remains the leading cause of death due to cancer in women [39]. Despite the fact that breast cancer incidence has increased over the years, mortality in the disease in many developed countries has remained constant or has even decreased [41].

Breast cancer incidence shows a striking regional variance. It is lowest in Africa and some parts of Asia, somewhat higher in Latin America, and highest in industrialised countries. Moreover, incidence is inversely associated with latitude – the further away from the equator, the higher the risk of breast cancer [39].

Breast cancer biology

The initiation of breast cancer is a cellular, multi-step genetic process, in which a series of defences must be overcome, leading to the classic malignant triad of growth, invasion and metastasis. Normal and malignant breast tissue is regulated both by systemic sex hormones, such as estrogens and progesterones, and by auto- and paracrine growth factors, such as epidermal growth factors (EGF), fibroblast growth factors (FGF) and insulin like growth factors (IGF). Hence, breast cancer cells are in intense interaction with their surroundings. Thus, breast cancer, perhaps more than any other cancer, is a systematic disease [42].

More than 95% of breast cancers are epithelial tumours, arising from the milk-producing glands (lobular carcinoma) or the draining ducts (ductal carcinoma) [42]. The current WHO-classification recognises six major histological types. Apart from the lobular and ductal types these also include phyllodes tumours, which are related to sarcomas, and medullary, mucinous and tubular types [43]. Besides type, *grade* also incorporates a histologic determination, which usually includes mitotic counts, the extent of tubule formation and the degree of nuclear atypia [44].

Modern molecular markers in breast cancer such as the expression of estrogen and progesteron receptors, p53, cathepsin-D, KI-67 and HER-2 receptors can aid in determining prognosis, guiding therapy, predicting the response to therapy and the risk of recurrence, and can be used in research. Stage and grade, however, still remain the most important determinants of survival in breast cancer [45].

Metastasis follows a clear pattern, and usually occurs first in the ipsilateral axillary lymph nodes. Lymph drainage usually passes one lymph node first, the *sentinel node,* which is the rationale of the current surgical technique in the axilla (see below). Distant metastasis can occur in bone and the liver and signifies incurable disease [42].

Stage refers to the clinical and/or pathological extent of disease, and is classified according to the International Union against Cancer (UICC/AJCC) classification [46]- T – tumour size, N – presence and number of lymph node metastases, and M – presence or absence of distant metastasis, such as liver and bone.

Diagnosis of breast cancer

The most common presenting symptom is a lump in the breast, but changes in size or shape of the breast, bleeding or excretion from the nipple, ulceration of the skin or enlarged nodes are sometimes the first signs and symptoms of breast cancer. The cornerstone in obtaining a correct diagnosis is triple assessment, which involves clinical examination, imaging such as mammography and ultrasound, and pathology. This enables a confident diagnosis in 95% of cases [42].

Some countries offer mammographic screening for women from around 40–50 years up until about 65–75 years of age. Randomised clinical trials conducted in the 1970s, with recent follow-ups, have shown a clear reduction in mortality in screened vs. unscreened [47, 48].

Treatment of breast cancer

The cornerstone of treatment is surgical excision of the primary tumour, either as a breast-preserving procedure, or with removal of the whole breast, mastectomy. With either procedure, immediate or late reconstructive surgery aiming at restoring the normal appearance of the breast can be offered. After breast-conserving surgery, radiation therapy to the remaining breast is usually recommended [42]. An operation to determine axillary nodal status is routinely performed, currently with the sentinel node technique. Pre-operatively, radioactive material is injected close to the tumour in the breast. During the operation, a blue dye is injected peritumourally. The radioactivity and the dye travel with the lymphatics and accumulate in one or a few lymph nodes. With radioguidance, these lymph nodes are excised and sent for immediate pathologic analysis. In the absence of nodal metastasis, no further axillary operation is performed. When present, a formal axillary lymph node dissection is carried out [42].

If the breast cancer is estrogen-receptor positive, therapy with anti-estrogens such as tamoxifen and aromatase inhibitors is recommended. The human epidermal growth factor receptor 2 (HER-2), a tyrosine kinase, is involved in breast cancer progression. Breast cancers that are positive for HER-2 can be treated with trastuzumab (Herceptin®), a recombinant humanised monoclonal antibody which targets HER-2 [49]. Chemotherapy is indicated mainly for premenopausal women with breast cancer [42].

Prognosis

If the tumour can be resected surgically and there are no signs of metastasis, prognosis is excellent. Even with limited axillary metastasis, there is a good chance of long-term survival. With distant metastasis, the disease is incurable. However, for all stages, a risk that breast cancer will recur will remain for the rest of the patient's life [45], which is in contrast to most other cancers, which are considered cured if there is no recurrence within five years of completed treatment. However, the biologic behaviour of breast cancer is remarkably individual, with patients with disseminated disease sometimes surviving for years, whereas others rapidly succumb.

Risk factors for breast cancer

Age is the most important risk factor. Before age 30, breast cancer is exceedingly rare. Incidence increases until the age of 80. When grouped together, women older than 65 years of age have a 5.8 fold increased risk of breast

cancer as compared to those younger than 65 years of age [33].

Having a mother or a sister with breast cancer increases breast cancer risk four-fold [33]. In the 1990s, two major susceptibility genes for breast cancer, BRCA1 and BRCA2 were identified [50, 51]. Women with germ line BRCA1/BRCA2 mutations have an estimated life-time risk of breast cancer of 65% and 45%, respectively [52]. However, the majority of familiar breast cancer cases are not due to mutations in these genes. Rather, breast cancer susceptibility is polygenic: several genes, each with a small effect, contribute to breast cancer risk. Technological advances have made it possible to analyse hundreds of thousands of single nucleotide polymorphisms (SNPs, which are DNA sequence variations occurring within a single nucleotide). These genome-wide association studies have identified novel breast cancer susceptibility loci, pointing to further plausible causative genes [53].

Previous benign breast disease, exposure to ionising radiation, and dense breast parenchyma as defined by mammography are risk factors associated with a two to four times increased incidence. Early menarche, late first full-term pregnancy, nulliparity, late menopause and exposure to HRT are all associated with a doubled risk [33]. Users of oral contraception have a higher risk as compared to non-users, but this risk declines when stopping, and in ten years returns to that of non-users [54].

High incidence has also been reported for obese postmenopausal women and for women in affluent socio-economic groups. Breastfeeding, early oophorectomy and physical activity are all associated with a reduced incidence of breast cancer [33, 55, 56].

Diets with a high glycemic index and alcohol consumption have been associated with a very modest but statistically significant increase in breast cancer risk [33, 57]. Anthropometric factors also affect breast cancer risk and a high BMI is a risk factor for breast cancer in postmenopausal women [33, 34].

Some of these risk factors also influence breast cancer behaviour, i.e. aggressiveness. For instance, it was found that users of HRT had an increased risk of breast cancer, but this increase was mainly seen in small, low-grade tumours without metastasis, i.e. tumours with better prognosis [58]. Importantly, some risk factors might interact with each other, synergistically increasing or decreasing breast cancer risk. For example, the relative risk associated with alcohol consumption increases as a function of increased BMI, while the relative risk associated with BMI increases as a function of patient age. Several models have been developed to assess the interactive effect of multiple risk factors on overall patient risk [33].

Calcium metabolism

Extracellular calcium is the most tightly regulated ion in humans. Most of the calcium (99%) in the body is stored in the skeleton, which serves as a reservoir of calcium. Circulating calcium thus constitutes only a small fraction of total body calcium. In blood, approximately 47% of calcium is free (ionised), 46% is bound to different proteins, mainly albumin, and the rest is bound to small ions.

In its ionised form, calcium serves as an intracellular messenger that participates in muscle contraction, neurotransmission, enzyme and hormone secretion, cell cycling and gene expression. Thus, calcium controls a wide range of essential cellular functions [22]. The extracellular free calcium is tightly regulated by PTH and 1,25-dihydroxyvitamin D ($1,25OH_2D$).

Calcium-regulating hormones

Parathyroid hormone (PTH)

The parathyroids are small glands located close to the thyroid, hence their name. Usu-

ally numbering four, they sense the extracellular calcium level by means of the calcium-sensing receptor (CaSR) and in a negative feed-back adjust their secretion of PTH accordingly [59]. PTH acts on PTH-receptors (PTHR1) found mainly in bone and kidney and its net effect is an immediate increase in serum calcium [60]. Thus, PTH is the main regulator of short-term serum calcium levels. PTH has a half-life of only about three to eight minutes and is rapidly degraded in the circulation [61].

Vitamin D

The main source of vitamin D is endogenous synthesis in the skin where vitamin D_3 is produced from 7-dehydrocholesterol under the influence of ultraviolet radiation, e.g. sunlight; another source is through the diet, as vitamin D_2 (from plants) or vitamin D_3 (from animals), either in food or as supplements [62]. Vitamin D is further transformed through enzymatic steps, first in the liver, to 25OHD, and secondly in the kidney to the active form $1,25OH_2D$. Both 25OHD and $1,25OH_2D$ exist in D_2 and D_3 forms.

25OHD is currently considered the best marker of human vitamin D status, since it has a long serum half-life, about three weeks. 25OHD thus indicates vitamin D stores obtained from both ultraviolet irradiation and dietary intake over long periods [14].

Vitamin D is technically not a true vitamin since it can be produced endogenously. Moreover, the term *vitamin D* does not refer to one single substance, but rather to a family of biochemically related steroid molecules, all with different biological properties.

Active vitamin D, $1,25OH_2D$, exerts its effects by binding to vitamin D-receptors, VDRs. These are found in the cell nucleus, where the ligand-receptor complex interacts with genomic DNA and selectively induces transcription and expression of certain genes [63]. They are also found in the cell membrane, where they are localised in flask-like invaginations called caveolae. These membrane VDRs are responsible for the more immediate actions of $1,25OH_2D$, such as the enhancement of intestinal calcium absorption [63].

The relation between vitamin D and PTH

PTH increases conversion of 25OHD into $1,25OH_2D$ by acting on the renal enzyme 1-α-hydroxylase. Both 25OHD and $1,25OH_2D$ act on the parathyroids to suppress PTH-production [64], and $1,25OH_2D$ also decreases parathyroid cell proliferation [65]. Low levels of 25OHD lead to decreased intestinal calcium absorption, causing a secondary increase in PTH [66, 67], which can be lowered by orally substituting vitamin D [66]. Hence, PTH and vitamin D are physiologically inversely related to each other. Several cross-sectional studies have found statistically highly significant but rather weak inverse correlations between PTH and 25OHD levels, with relation coefficients (r) not exceeding –0.39 [68].

Calcitonin

The role of calcitonin, a 32-amino acid peptide secreted by the C-cells of the thyroid, in human calcium metabolism is unclear. Calcitonin inhibits bone resorption and is used therapeutically in diseases such as Paget's disease and hypercalcemia of malignancy [22]. Its physiological role in calcium metabolism in humans, if any, has been challenged since absence of calcitonin (for example, after thyroidectomy) does not seem to affect calcium metabolism [22].

Diet and calcium metabolism

Dietary calcium

About 70% of dietary calcium comes from milk and dairy products, mainly cheese [69].

Commonly, fruit juices, soft spreads, wheat flour, milk and milk products such as yoghurts are fortified with calcium [70]. Calcium is widely available as non-prescription multivitamin supplements, and is prescribed as prophylaxis and treatment for several conditions, most notably osteoporosis. In many countries, including Sweden, the recommended daily allowance (RDA) is about 900 mg/day [71], rising to 1200 mg/day for adolescents and the elderly [70].

Dietary vitamin D

Vitamin D occurs naturally as vitamin D_3, in some animal foods, especially fat fish [72]. Currently, many foods, such as cereals, milk, milk products, and fruit juices are fortified with vitamin D [72], either with vitamin D_2, manufactured from yeast ergosterol, or vitamin D_3. Studies indicate that vitamin D_3 is more effective than vitamin D_2 in terms of raising serum 25OHD levels [73–75] but current dietary and supplementary recommendations do not distinguish between the two [71, 72]. The RDA for vitamin D is around 400 IU (10 µg) in many countries [72], including Sweden [71]. Low 25OHD levels and high dietary phosphates decrease intestinal calcium absorption [76]. It has been suggested that increasing the amount of calcium ingested directly affects the metabolism and leads to corresponding lower serum levels of 25OHD [77].

However, despite large fluctuations in the ingested amounts of calcium and vitamin D, serum calcium remains within very narrow limits, due to the immediate effects of circulating PTH [22].

Calcium metabolism in normal breast tissue

Breast milk contains large amounts of calcium to supply the needs of the growing skeleton in the newborn. During lactation, the mammary gland excretes PTH-related peptide, PTHrP, which mediates the release of calcium from the maternal skeleton for transfer to milk, thus functioning as an accessory parathyroid gland [11]. In humans, PTHrP is composed of either 141 amino acids, or, due to alternative splicing, 139 or 173 amino acids. It shares considerable N-terminal sequence homology with PTH and acts on the same receptor [78]. It functions as a local autocrine or paracrine factor with several important physiological roles, including the regulation of chondrocyte growth and differentiation of the growth plates of developing long bones [78]. Studies in animals suggest that PTHrP also regulates mammary development [79]. It has similar physiologic effects as PTH in terms of calcium metabolism.

During lactation, an increase in $1,25OH_2D$ is also seen, probably mediated by PTHrP acting on renal 1-α-hydroxylase.

Calcium metabolism and female sex steroid hormones

Estrogens given perorally or parenterally decrease serum total and ionised calcium concentrations, and total plasma calcium rises at menopause [80]. Estrogen administration to postmenopausal women increases the concentration of serum $1,25OH_2D$, perhaps by increasing the conversion of 25OHD to $1,25OH_2D$ in the kidney [81].

One *in vitro* study showed that estradiol and/or progesterone stimulates PTH-secretion from human parathyroid tissue [82], but other experimental work found no evidence of estrogen receptors in bovine parathyroid glands nor in parathyroid adenomas [80]. Hence, there has been considerable disagreement regarding the effects of estrogen on the regulation of PTH-secretion; it has been reported that estrogen reduces the set-point for PTH release by calcium but also that estrogen has no effect on the relationship between calcium and PTH-secretion [83].

Perhaps physiologically more important is

the effect of estrogen on skeletal responsiveness to PTH. Under normal circumstances, PTH releases calcium from the skeleton, not by directly activating osteoclasts but rather through the stimulation of osteoblasts to secrete cytokines. These act on osteoclast progenitor cells and induce differentiation into mature osteoclasts, which resorb bone and release calcium [84]. Estrogen, via the estrogen receptor, blocks this PTH-stimulated osteoclast formation [84, 85]. This mechanism might be responsible for the calcium-decreasing effect of estrogen.

Disorders related to calcium, vitamin D and PTH

The two most common causes of clinical hypercalcemia are pHPT, primary hyperparathyroidism and HHM, humoral hypercalcemia of malignancy, respectively [86]. Some rare causes are granulomatous disorders, such as sarcoidosis, which produce $1,25OH_2D$. Hereditary defects in any of the calcium-regulating hormones, the most common of which is FHH, familial hypocalciuric hypercalcemia (which is usually due to a mutation in the CaSR [87]) can also, though rarely, be the cause of hypercalcemia. Renal failure can be complicated by secondary hyperparathyroidism, with or without hypercalcemia.

Primary hyperparathyroidism

Primary hyperparathyroidism is not uncommon and is usually due to a benign adenoma in one of the parathyroid glands. Sometimes hyperplasia of several glands is seen and very rarely parathyroid carcinoma is the underlying cause.

PHPT most commonly afflicts postmenopausal women; in these, the prevalence is about two percent, but the disease can occur in both sexes at any age [88].

Classically, pHPT used to present with renal stones, osteoporosis, and constipation. Today, most patients are diagnosed with milder disease [60]. Neuropsychiatric and cognitive symptoms, such as lowered mood, memory impairment, muscular weakness and fatigue are common presenting symptoms of pHPT [89]. The disease can have a mild and protracted course, but long-term studies suggest that pHPT carries an increased risk of osteoporosis, cardiovascular disease and possibly also cancer [90].

Treatment consists of surgical removal of the diseased gland(s). In experienced hands, this has a success rate of 95–99% with minimal morbidity and almost no mortality. Since symptoms are sometimes diffuse, the policy on whom to treat has been controversial. A recent NIH guideline states that only symptomatic individuals or those with serum calcium above 2.85 mmol/l should be offered surgery [89]. Others have advocated surgery for everyone with the disease, citing the excellent results achieved by surgery [91].

Humoral hypercalcemia of malignancy

A common complication of advanced cancer is hypercalcemia, so called humoral hypercalcemia of malignancy, HHM. This is usually caused by paraneoplastic production of PTHrP, and in fact, this condition led to the discovery of that peptide [92]. HHM is a common complication of advanced breast cancer, perhaps reflecting the physiologic importance of PTHrP expression in the lactating breast [93].

Vitamin D deficiency

Defining optimal vitamin D intake and serum 25OHD levels has been controversial [62]. Severe vitamin D deficiency causes rickets, a serious bone disease characterised by abnormal mineralisation. Until recently, recommendations on vitamin D intake and serum 25OHD levels were based on the amount

needed to cure rickets [94], which roughly corresponds to 25OHD levels of about 27.5 nmol/L [75].

However, recently, the importance of vitamin D for organs and systems outside the skeleton has been highlighted [63]. Researchers have employed several different methods to define sufficient vitamin D levels based on physiologic, clinical and experimental data. For instance, it was shown that intestinal calcium absorption is optimal at 25OHD levels of approximately 80 nmol/L [95]. Another way of defining 25OHD-sufficiency is by examining the optimal concentration for the enzyme responsible for converting 25OHD into the active metabolite, $1,25OH_2D$. This concentration, the K_m, is around 100 nmol/L [63].

Yet another way has been through measuring PTH. Since PTH is inversely correlated with 25OHD, an increase in 25OHD leads to a decrease in PTH, up to a 25OHD level of about 78 nmol/L [96], where PTH levels plateau.

Using multiple clinical outcomes, such as bone and oral health, fall tendency, and prevention of colon cancer, a recent review concluded that 25OHD levels of at least 75-100 nmol/L were required [97].

Thus, based on physiological, clinical and experimental reasoning, several authors agree that 25OHD levels of at least 75 nmol/L are needed [98] for vitamin D sufficiency. With this definition, vitamin D deficiency is widespread [62, 99], affecting the majority of Swedes, at least in winter [100]. Risk factors for vitamin D insufficiency include poor diet and lack of exposure to sunshine. Risk groups thus include the elderly, institutionalised people, and immigrants. Treatment is simple, by oral substitution. There is currently no consensus on optimal dose but most authors conclude that substantially higher intakes than those currently recommended are needed [94, 99].

Calcium, vitamin D, PTH and breast cancer

Experimental studies

Calcium

Increasing calcium concentrations decreases proliferation and increases differentiation in breast cancer cell lines *in vitro* [13]. The calcium-sensing-receptor, CaSR, is expressed both in normal [23] and malignant breast cells [24] and its expression seems to be correlated with skeletal metastasis [24, 25], which could indicate a relationship between serum calcium and tumour aggressiveness. Extracellular calcium downregulates the estrogen receptor in breast cancer cells, and this is mediated by the CaSR [101], which could also imply an association between serum calcium and tumour aggressiveness. It has also been reported that calcium releases the growth inhibition of $1,25OH_2D$ on breast cancer cells [102], indicating a possible tumour promoting effect of extracellular calcium.

Vitamin D

Both the vitamin D receptor, the VDR and the converting enzyme, the 1-α-hydroxylase, are expressed and dynamically regulated in the normal mammary gland [4]. Experimental studies have shown that $1,25OH_2D$ can inhibit cellular proliferation, induce apoptosis and inhibit angiogenesis in both normal and malignant breast cells [13]. More specifically, $1,25OH_2D_3$ has been shown to reduce the proliferation of MCF-7 and BT-20 cell lines regardless of their sex steroid receptor status [103]. Experiments on induced mammary tumours in Sprague Dawley rats found that $1,25OH_2D_3$ given at non toxic doses reduced the tumour proliferation [103]. Furthermore, studies with mice lacking VDRs, so-called knock-outs, showed that these had abnormal ductal morphologic fea-

tures, increased incidence of preneoplastic lesions and accelerated mammary tumour development [4]. 1,25OH_2D has also been shown to arrest cell cycling, thus inhibiting proliferation [104]. However, doses needed to achieve these antitumoral properties are much higher than those usually found in the circulation [4].

PTH and PTHrP

PTH and PTHrP share the same receptor, PTH-receptor 1, PTHR1 [105], which is expressed both in normal and malignant breast tissue [106]. The expression of PTHR1 in breast cancer cells promotes their autocrine proliferation [21]. Both PTH and PTHrP have carcinogenic and tumour promoting effects [19–21, 106–108]. PTHrP is also related to breast cancer aggressiveness in that its expression in breast cancer predicts future bone metastasis [109, 110] and correlates with poor prognosis [111].

PTHrP is often expressed in bone metastases [112]. Extracellular calcium increases the production of PTHrP in breast cancer cell lines, and it is been suggested that serum calcium participates in a vicious circle, where PTHrP-induced bone resorption raises serum calcium, which then acts to further increase PTHrP production [24].

Epidemiological and clinical studies

Calcium

Studies on dietary calcium and breast cancer incidence have generally found negative correlations. Some of these studies have been hospital based case-control studies, with potential confounding, and others have been small and/or not controlled for known risk factors of breast cancer, reviewed by Cui and Rohan [13]. This review concluded that the potential association between dietary calcium and breast cancer risk is uncertain and needs further investigation.

Two cohort studies found that serum calcium and untreated hypercalcemia were both positively associated with an increased risk of death during a follow-up of 10.8 and 14 years respectively [113, 114].

Prior to the current thesis there has been no prospective cohort study on serum calcium and breast cancer incidence.

Vitamin D

Ecological studies have found higher breast cancer incidence in sun poor regions [115] and inverse relations between sun exposure and breast cancer risk [13]. On the other hand, studies comparing intake of vitamin D with breast cancer risk have been inconclusive [13].

Cross-sectional case-control studies have indicated a protective effect of serum 25OHD levels in breast cancer [116–118]. Three prospective case-control studies have found a weak, statistically non-significant negative association between 25OHD levels and breast cancer risk [15–17]. On the other hand, a randomised trial found a reduction of breast cancer incidence in postmenopausal women treated with vitamin D and calcium supplements [9]. In that trial, there was also a statistically significant negative association between 25OHD levels and cancer risk.

The risk of breast cancer in relation to 1,25OH_2D has been investigated in at least three prospective studies [15, 16, 119], with weak and inconsistent findings, none of which were statistically significant.

PTH

At least four record-linkage studies have found a weak positive correlation between risk of breast cancer and primary hyperparathyroidism [1–3, 18]. Three of these [1, 2, 18] linked data on surgery for pHPT with cancer inci-

dence in the Swedish Cancer Registry. Analyses were based on a large number of pHPT patients, ranging from 4,163 to 9,835. Standardised incidence ratios (SIRs) for breast cancer in treated pHPT were calculated and found to be 1.27–1.44. One study [3] linked pHPT diagnoses in the Danish national inpatient registry with the Danish national cancer registry and found an SIR of 1.43.

Materials and methods

The City of Malmö – population at risk

Malmö is the third-largest city in Sweden, with a population of 280,801 as of 1st January, 2008 [120]. It is situated in one of the regions with the highest breast cancer incidence in Sweden – 178/100,000 as compared to the average in Sweden of 154/100,000 [40]. Incidence increased when mammography was introduced in a screening trial in 1976, and again when screening was made available for all women aged 50–69 years in 1990 [40].

The Malmö Preventive Project – MPP

The Malmö Preventive Project was established in 1974. It invited participation by entire birth-year cohorts of Malmö residents (in females the birth-year-cohorts of 1926, 1928, 1930, 1931, 1932, 1934–1936, 1938, 1941, 1942 and 1949). It was directed against cardiovascular diseases, diabetes mellitus and alcohol abuse. In an outpatient clinic, participants filled out a comprehensive questionnaire containing 260 questions using a computer. Questions centred on family history of cardiovascular disease, hypertension and diabetes; smoking habits and signs of high alcohol consumption; physical activity and socioeconomic factors. The questionnaire was revised several times during the project.

Subjects had their weight and height measured when wearing light indoor clothing and BMI was calculated (kg/m^2). Blood pressure and pulse rate were measured twice after ten minutes rest in the supine position. Routine blood tests were taken, including electrolytes, liver enzymes, hemoglobin, creatinine, triglycerides and cholesterol.

Individuals at risk (for example, smokers, those who were obese, those with high alcohol consumption) and those with signs and symptoms of disease were offered individualised advice and treatment in a nearby unit, with referral to specialists when needed [121].

When the department closed in 1992, 10,902 women had been examined [122] corresponding to an overall attendance rate of 70%.

The Malmö Diet and Cancer Study – MDCS

This cohort was set up as an epidemiological project to study the association between dietary factors and cancer incidence [123]. Between 1991 and 1996 it recruited men and women in Malmö born between 1923 and 1950. Out of a population of 74,138 subjects, 68,905 eligible individuals were invited. A total of 28,098 respondents (40.8%) completed baseline examination, which included dietary assessment, a self-administered questionnaire, anthropometric measuring and collection of blood samples. The questionnaire assessed socioeconomic factors and life-style factors, medications, and previous disease, but also included questions on subjective well-being, weight changes and physical activity [124]. In all, 17,035 women completed all study parts [124].

Dietary assessment consisted of a menu book, a diet history questionnaire and a dietary interview. In the menu book, participants recorded meals, beverages and dietary supplements over seven consecutive days. Data from the menu book, the questionnaire and

interview were coded and converted into nutrient intake data [125]. Height, weight, waist and hip circumferences were measured by a trained nurse. BMI was calculated as kg/m^2.

Even in the planning stage, great care was taken to ensure proper registration, maintenance and handling of biological specimens linked to the cohort, such as blood and serum samples [126], which were stored at –80°C [127].

Study populations

Papers I, II and III are all based on the MPP cohort, which in total comprises 10,902 women. In papers I and III, information on reproductive factors including menopausal status was considered necessary for analyses. Items in the baseline questionnaire focussing on reproductive factors were introduced in April 1983. Women included in the project from this date and onwards and who had given information on their menopausal status were selected for analysis in papers I and III (*n*=8,051). Serum calcium had been measured in 8,004 of these. A total of 157 women with prevalent invasive breast cancer at baseline were excluded. Papers I and III are therefore based on 7,847 women.

In paper II, all women examined following April 1983 were selected (n=8,161), accepting the fact that some of these had no information on menopausal status. Out of these, 8,114 had information on serum calcium levels and this group was used for analysis in paper II.

In paper IV, 766 incident breast cancer cases were identified within the MDCS, and 764 out of them had blood samples drawn at baseline. Incidence density matching, using age as the underlying time scale, was used in order to select one control for every case. This meant that individuals with incident breast cancer, i.e. cases, could themselves serve as controls for cases which had been diagnosed earlier. Matching criteria were calendar time at inclusion (+/– 15 days), menopausal status (pre- vs. peri-/post) and age at inclusion (+/– 2 years).

The narrow time span for time of inclusion, i.e. time of blood donation, was given high priority, as 25OHD levels have a marked seasonal variation. In all, 760 case-control pairs were exactly matched according to the above criteria. Age at baseline was relaxed to +/– 3 years in 2 pairs, and to +/– 4 years in 2 pairs.

Controls were originally matched to cases at a 2:1 ratio, but only one control for each case was used in the laboratory analyses. The rationale for matching on two controls was to be able to use another control when there was no serum available for the first. Following sample retrieval, thirteen individuals had insufficient amounts of serum. Nine were cases and could not be replaced, leaving four controls. One pre-matched control was already part of another case-control pair. In all, three new individuals replaced three original controls. Finally, 1,483 unique individuals were included in the study, corresponding to 1,528 observations (764 case-control pairs).

Laboratory analyses

Investigations in papers I–III are based on results of blood samples drawn and analysed at inclusion. In the morning, after an overnight fast, all subjects gave a blood sample, which was centrifuged and analysed immediately. Calcium was measured photometrically by the laboratory at the Dept. of Clinical Chemistry, University Hospital of Malmö, on a PRISMA multi-channel autoanalyser (Clinicon AB, Bromma, Sweden). The coefficient of variation (CV) was 1.52% [128]. The reference value for adult women for serum calcium during this period was 2.20–2.60 mmol/L.

In paper IV, serum from cases and controls was retrieved from the MDCS biobank. Samples had not been previously thawed. Serum was analysed for 25OHD$_2$, 25OHD$_3$, PTH, calcium, phosphate, creatinine and albumin. Case-control pairs were analysed in a random

sequence with regard to the case-control order and with regard to time of baseline examination. Cases and controls in the same pairs were always examined in the same batch, except for one control that had to be replaced, as described above.

$25OHD_2$ and $25OHD_3$ were analysed with high pressure liquid chromatography (HPLC) and PTH with the Immulite® 2000 Intact PTH immunoassay (Diagnostic Products Corporation, Los Angeles, CA). Total calcium was analysed by neutral carrier ion-selective electrode [129], albumin by rate immunenephelometry [130], and phosphate by a colorimetric method by complexing with amonniummolybdate and creatinine by the Jaffé method. These analyses were carried out with the Synchron LX System (Beckman Coulter Inc., Fullerton, CA.)

All laboratory measurements were normally distributed except for PTH. CVs were for $25OHD_2$ 8.0% at 65 nmol/L and 6.8% at 190 nmol/L, for $25OHD_3$ 8.5% at 70 nmol/L and 7.1% at 210 nmol/L, for PTH 4% at 5.9 pmol/L and at 40.3 pmol/L, for calcium 2% at 2.00 mmol/L and at 3.10 mmol/L, for albumin 4% at 25 g/L and 2% at 48 g/L, for creatinine 12% at 34 µmol/L and 4% at 129 µmol/L, and for phosphate 3% at 0.66 mmol/L and 5% at 2.5 mmol/L. The laboratory of the department of Clinical Chemistry at Malmö University Hospital is accredited by Swedac (The Swedish Board for Accreditation and Conformity Assessment) and takes part in the external quality assurance program of Instand e.V., Düsseldorf, Germany.

Cancer endpoints and vital status

The Swedish Cancer Registry was set up in 1958. By law, all malignant tumours and certain benign tumours diagnosed in Sweden must be reported to the registry [40].

Breast cancer cases, invasive and in situ, were retrieved by record linkage with the Swedish Cancer Registry and the Southern Swedish Regional Cancer Registry. The national register is complete, with a one-year delay. The Southern Swedish Regional Tumour Registry has provided up-to-date information on cancer incidence in the south of Sweden since it was established in 1977 [131].

All deaths and causes thereof must be reported to the Swedish Cause-of-Death registry (SCDR), which was set up in 1911. It contains, among other things, the deceased individual's name, the civil registration number, cause of death, and date of death [40].

The Swedish Cancer Registry and the Swedish Cause of Death Registry have been validated and found to have a completeness of about 99% [131]. The SCDR has a delay of about two years, and up-to-date vital status was also retrieved from the Population Registry.

Histopathologic examinations

In paper III, tumour samples were re-evaluated by two senior pathologists. Histologic type was determined according to the WHO classification [43]. Tubular formation, nuclear atypia and mitotic index were determined according to the Nottingham classification, as described previously [44], where each parameter is graded from one to three. The presence or absence of axillary lymph node metastasis, and tumour size in mm were determined from pathology reports.

Statistical methods

Multivariate analysis was used to determine relative risks (RR) and odds ratios (OR) of breast cancer in different quartiles of calcium (papers I and IV) and 25OHD and PTH (paper IV). Quartile cut-points for these analytes were based on the distribution for women in the study cohort excluding those with prevalent cancer of any site (not including cervical

cancer in situ) in paper I, and in controls in paper IV. In paper III, the cohort was dichotomised (due to having small subgroups) based on serum calcium levels using the same criteria as in paper I. Cox's proportional hazards analysis was used to estimate relative risks of breast cancer in different calcium quartiles in paper I and breast cancer subgroups in paper III. In paper IV, unconditional and conditional logistic regression was used to calculate odds ratios with 95% confidence intervals (CI). Potential confounders and known risk factors of breast cancer were introduced as covariates. Missing covariates were coded as separate categories for categorical factors, and means for all subjects with data were used for subjects with missing values on albumin, creatinine and phosphate. Separate analyses were made in pre- vs. peri-/postmenopausal women and in different strata of BMI, i.e. BMI < 25 vs. BMI ≥ 25 (overweight and obese women). All analyses were repeated, excluding cases diagnosed within two years following baseline examination.

In paper II, the association between serum calcium and reproductive and selected lifestyle factors was investigated. Means of serum calcium were calculated in different categories of the studied factors. An ANOVA and a Student's t-test with Bonferroni's correction were used to test differences in calcium levels between different categories of the studied factors. All tests were two-sided and a p-value less than 0.05 was considered statistically significant.

All women were dichotomised into 'low' (≤2.34 mmol/L) and 'high' (≥2.35 mmol/L) calcium levels, the median of the study cohort. Then, ORs with 95% CIs were calculated for 'high' vs. 'low' calcium levels in relation to the studied factors, using an unconditional logistic regression analysis. The second model was adjusted for age and a final model included all studied factors. Calcium levels were approximately normally distributed and the relation between different factors and calcium levels was further investigated using multiple linear regression analysis. All categorical variables in the linear regression analysis were transformed and entered as multiple categorical variables. Partial regression coefficients (β_i) with 95% confidence intervals, adjusted for all other factors, were reported.

Statistical analyses were performed with SPSS versions 13.0 through 16.0 (SPSS Inc. Chicago, Ill.).

Ethical considerations

Ethical approval was given for all projects included in the thesis (LU 51-90, LU 639-03, Dnr 652/2005 and Dnr 23/2007). Participants in the MPP were not recruited primarily for research, but with the goal of reducing their risk and treating disease. The MDCS had a primary research objective and subjects gave informed consent at entry. Additionally, for the purpose of the present analyses, former participants in the MPP and the MDCS were informed of the aim and of the possibility of withdrawing from the study, by advertising in local newspapers.

In paper I–III, no new information on subjects was generated, and results were not deemed to have implications on an individual level. Thus, it can be considered that participants suffered no risk of harm due to these studies.

In paper IV, the situation was different as blood donated by subjectively healthy individuals was analysed and new information was obtained. As expected, there were incidental findings, with blood chemistry measurements outside the reference range, potentially indicating disease. It was decided not to inform study participants of these incidental findings. This was based on current ethical recommendations [132]. There were several reasons. First, these incidental findings related to a situation 12–17 years ago, and their current relevance to participating individuals could be questioned. Second, a proportion of all analy-

ses in a healthy population are expected to be outside the reference limit, for example the reference limits for laboratory analyses often include only 95% of all individuals. That is, a false-positive rate of five percent can be expected. Third, participants in the MDCS agreed to participate in a research project; they were not informed that they were to be contacted considering every subsequent analysis. Fourth, since individuals only donated blood once, and the sensitivity and specificity of the tests were not 100%, this would require additional contacts with former participants for repeated tests. Repeated contacts, the risk of false-positive findings, and additional medical examinations could, hence, have led to psychological and physical distress.

Results

Paper I

In premenopausal women, breast cancer incidence was found to be negatively associated with calcium levels and RRs (95% CI) in the 2nd, 3rd and 4th calcium quartile as compared to the 1st were 0.92 (0.65–1.31), 0.88 (0.59–1.30) and 0.56 (0.32–0.99). In peri-/postmenopausal overweight women, breast cancer incidence was positively associated with calcium levels, the RRs in the 2nd, 3rd and 4th calcium quartiles as compared to the first were 2.74 (1.25–5.98), 3.10 (1.44–6.68), and 2.72 (1.24–5.94).

Paper II

Calcium levels were strongly and inversely associated with use of oral contraception and use of HRT, with correlation coefficients (95% CI) of –2.17 (–3.05 to –1.30) and –4.19 (–4.77 to –3.62), respectively. Peri-/postmenopausality was positively associated with calcium levels, with a correlation coefficient of 3.88 (3.35–4.40). Calcium levels also showed a weak but statistically significant positive association with nulliparity, BMI<20 and BMI>25 and baseline examination in spring and autumn.

Paper III

In women with BMI≥25, calcium was significantly associated with aggressive tumours as determined by severe nuclear atypia, with an OR (95% CI) of 2.06 (1.10–3.86) for 'high' (above median) calcium as compared to 'low' calcium. This was also seen for mitosis and tubular formation, but for these subgroups, the relative risk did not reach statistical significance.

Calcium was associated with a significantly higher risk of nodal metastasis vs. no nodal metastasis in premenopausal women; the OR (95% CI) for 'high' as compared to 'low' calcium was 1.88 (1.04–3.38). Similarly, in premenopausal women the heterogeneity analysis revealed that 'high' calcium was associated with a higher risk of T1N1 tumours as compared to 'high' calcium in relation to T1N0 tumours.

Paper IV

In the overall analysis, there was a weak negative association between $25OHD_3$ levels and breast cancer risk, but this association was not statistically significant, and it was less pronounced in the adjusted analysis. The association between total 25OHD ($25OHD_2$+D_3) and breast cancer was even weaker. The association between PTH levels and breast cancer risk was close to unity. Calcium was positively associated with breast cancer risk in the multivariate analysis, but this association did not reach statistical significance. ORs were also similar in matched and unmatched analyses, indicating that unmatched analyses were appropriate.

When stratifying for menopausal status and BMI, there was a significant positive asso-

ciation between serum calcium and breast cancer in overweight and premenopausal women, respectively.

In women with $25OHD_3$ <75 nmol/L, PTH and calcium were positively associated with breast cancer risk, but confidence intervals were wide and statistically non-significant. There was a weak, statistically non-significant, negative association between $25OHD_3$ and breast cancer in women with PTH levels above the median. Risk estimates related to different 25OHD and PTH quartiles were similar in analyses stratified for calcium levels below vs. above the median.

General discussion

The current thesis suggests that calcium levels affect breast cancer risk, and that this risk is modified by menopausal status and obesity. Moreover, serum calcium levels are clearly associated with age, BMI, use of OC, HRT, and menopausal status – all established risk factors for breast cancer.

There may be a weak, inverse association between 25OHD levels and breast cancer, but this association was not statistically significant. There was no association between PTH levels and breast cancer risk.

Results on the relationship between serum calcium and breast cancer were most concordant in overweight women (BMI≥25). In premenopausal women, there was a negative association between serum calcium and breast cancer risk in the MPP cohort (paper I), and a positive association in the MDCS case-control study (paper IV). Study populations differed in some accounts: MPP subjects were younger at inclusion, had a longer mean time from inclusion to diagnosis of breast cancer and were younger at diagnosis than subjects in the MDCS. This may be of special interest where subjects are defined as pre/postmenopausal at baseline.

Serum calcium levels and reproductive factors

Both younger (40–45 years) and higher age groups (>55 years) had higher calcium levels as compared to women aged 45–50 years, even when adjusting for menopausal status, suggesting that age has an independent influence on calcium levels. BMI was also significantly associated with serum calcium levels, with lean and overweight women having higher calcium levels than women with BMI between 20 and 25.

The present work also confirmed previous studies on reproductive factors and calcium levels, with an inverse association between conditions characterized by high estrogen levels and serum calcium levels. This has been found in studies on menopausal status [26, 133–135], phases of the menstrual cycle [136], use of OC and HRT [133, 137] and pregnancy [138]. Experimental studies also indicate that serum calcium levels drop when estrogens are administered [80]. The exact mechanisms remain unclear, but the effect might be mediated through a change in skeletal sensitivity to PTH [84, 85, 139–141].

Serum calcium and breast cancer

Calcium is an important intracellular messenger, involved in processes related to proliferation, apoptosis and cell signalling. The calcium-sensing-receptor, CaSR, is expressed both in normal [23] and malignant breast cells [24] and its expression is correlated with skeletal metastasis [24, 25]. Increasing levels of calcium can, in experimental models, increase cell differentiation, decrease proliferation, induce apoptosis and down-modulate invasion [142–144], all of which would have tumour protective effects. On the other hand, a case-control study found a positive association between calcium concentrations in benign breast tissue and subsequent breast cancer risk [145],

and one study found that increasing calcium concentrations released the growth inhibition of $1,25OH_2D$ on breast cancer cells [102]. Thus, data from *in vitro* studies are not consistent regarding calcium and tumour growth and further investigation is warranted of the present findings that serum calcium levels are positively associated with breast cancer risk in overweight and/or postmenopausal women, and with breast cancer aggressiveness in premenopausal and/or overweight women.

Vitamin D and breast cancer

The biologically active form of vitamin D, $1,25OH_2D$, is a steroid hormone that binds to vitamin D receptors, VDRs [63] which are found in both normal and malignant breast tissue. $1,25OH_2D$ has been shown to inhibit cellular proliferation, induce apoptosis and inhibit angiogenesis [13], mechanisms that may link vitamin D to tumour protective effects.

The risk of breast cancer in relation to $1,25OH_2D$ has been investigated in at least three studies [15, 16, 119], with all studies finding no statistically significant associations. However, $1,25OH_2D$ has a short half-life and shows great intra-individual variation; thus it is generally considered that 25OHD better mirrors physiologic vitamin D status [14].

Cross-sectional case-control studies have indicated a protective effect of 25OHD in breast cancer [116–118]. A randomised trial found a reduction of breast cancer incidence in postmenopausal women treated with vitamin D and calcium supplements [9]. In that trial, there was also a statistically significant negative association between 25OHD levels and breast cancer risk.

The present prospective study (paper IV) found a negative association between 25OHD levels and breast cancer risk, but the association was weak and not statistically significant, which was in line with previous prospective investigations [15–17]. It is possible that neither of these studies, including the present, had sufficient statistical power to detect a true negative association between 25OHD and breast cancer risk and that a real modest inverse relation exists. Possibly, meta-analysis by pooling of data might clarify this issue.

PTH and breast cancer

Experimental studies suggest that PTH has carcinogenic and tumour promoting effects [19–21]. At least four record-linkage studies have found a positive association between risk of breast cancer and primary hyperparathyroidism (pHPT), a condition with high PTH and often high serum calcium levels [1–3, 18]. Hence, a positive association between PTH levels and breast cancer risk could be expected, but no such relation was observed in the present study (paper IV). One reason for this discrepancy could be that PTH only causes an increased risk of breast cancer at levels clearly above the normal range, as is seen in most patients with pHPT. In the present study, only eight cases and six controls had both PTH and calcium levels above the reference range, which made statistical analysis impossible in these cases. Approximately nine percent of both cases and controls had PTH levels above normal, signifying possible pHPT. In an additional analysis calculating OR for breast cancer in these subjects, compared to those that had PTH values below the upper reference limit, the OR was close to one and did not have statistical significance.

Methodological issues

Exposure and endpoint measurements

It may be questioned whether it is appropriate to use a single determination for levels of calcium, 25OHD and PTH.

Under normal physiological conditions, total calcium is very stable. Both short-term

[146] and long-term [147] intra-individual variation are low. Even though serum calcium levels rise with menopause [26, 148] there seems to be significant 'tracking', i.e. the ranking of calcium levels between individuals tends to remain the same before and after menopause [135]. Although inter-individual differences in absolute values for serum calcium are low, these differences are still considered important when large groups are compared. Thus, it can be argued that a single measurement of serum calcium is a useful marker for differences with regard to calcium homeostasis.

It has been claimed that free (ionised) calcium provides the best indication of calcium status because it is biologically active and tightly regulated by calcium-regulating hormones. Total calcium levels are affected by plasma protein levels, notably albumin. In the MPP cohort (papers I–III), adjusting for serum albumin was not possible since albumin levels were only known for about a quarter (n=2,048) of the study population. However, total calcium has been considered a good measure of calcium homeostasis in outpatients and healthy individuals where albumin will be expected to be in the normal range [149]. Albumin was normally distributed among those with known albumin levels, and only seventy-five women (3.7%) had an albumin level outside the normal reference range (36–45 g/L). All samples were also collected in a standardised manner, which minimised differences in albumin levels due to fasting status or diurnal variation [113]. Following this, total serum calcium can be considered a useful and valid measurement of calcium status in this study population.

In the MDCS cohort (paper IV), correction of serum calcium according to albumin was possible since albumin levels were known for practically all subjects. However, there are several correction methods and no single one has been proved to be superior to the other. Instead, it was decided to adjust for albumin (along with creatinine and phosphate, which may also affect calcium levels) as continuous covariates in the multivariate analyses.

Regarding 25OHD, it is reassuring that a recent cohort study measured 25OHD on two occasions, three years apart, and found a high correlation between levels [150]. It was also possible to measure $25OHD_2$ and $25OHD_3$ separately, improving precision. Cases and controls were closely matched to calendar time for blood sample, minimising variation in 25OHD levels due to sun exposure.

Data on PTH has been scarce but recently, two publications have addressed short-term (up to six weeks) variation. Intra-individual total, i.e. analytical plus biological, CV in serum PTH levels was about 25% [151, 152]. PTH also shows a relatively large circadian fluctuation with a two-fold difference in nadir to peak concentrations [152]. In the MDCS, the time of day for blood sampling had not been recorded.

If these reported variations are correct, biological intra-individual variations of PTH levels are quite high, which may be expected to lead to a non-differential misclassification of PTH levels. This may, hence, lead to an attenuation of true risks in the statistical analysis. Considering the findings in the present study, it is possible that this potential misclassification has obscured true, underlying associations between breast cancer risk and PTH levels in paper IV.

Incomplete follow-up and poor quality of endpoint data may affect the results. However, the Swedish Cancer Registry and the Swedish Cause of Death Registry have been validated and found to have a completeness of about 99% [131].

Representativity

It may be asked whether breast cancer cases in these cohorts can be considered representative of the whole breast cancer population. The study cohorts mainly comprised middle-aged women. In the MPP, 30% and in the MDCS, 60% of the women invited to the health ex-

amination did not attend. As there was no information about exposure to the studied risk factors in women outside this cohort, absolute risks and incidence rates may not be applicable to all age groups or to the general population. However, as there was a wide distribution of calcium, 25OHD and PTH levels, it was possible to make internal comparisons between subjects with low and high values respectively, i.e. to estimate relative risks. Results were probably not considerably affected by selection bias.

Confounding

In all papers, there was information on most established risk factors for breast cancer, and results were similar when adjusting for these factors. Thus, results were probably not confounded by most known risk factors for breast cancer.

We could not adjust for heredity in any of the papers since there was no information on this factor. The quality of the variable for physical activity in the MDCS is currently being validated, and it was decided not to include this variable in the analyses. In papers I, II and III, it would have been valuable to have information on factors related to calcium metabolism, such as 25OHD, PTH and albumin levels, dietary information and sun exposure.

In paper IV, it was possible to adjust for factors related to calcium metabolism, such as 25OHD, PTH, creatinine, albumin and phosphate. Dietary information was not included in the multivariate analysis, the rationale being that a potential association between dietary intakes of vitamin D and calcium and breast cancer risk is probably mediated by the serum levels of these factors. This would make it inappropriate to include dietary intake in the same model as the serum levels.

In all papers, multiple analyses were done, and chance findings cannot be completely ruled out. However, narrow confidence intervals, large cohorts, many cases and concordant findings, at least concerning the association between breast cancer and serum calcium in women with BMI>25 suggest that the findings are not due to chance alone.

In all papers, associations between outcomes and predictors (for example breast cancer and serum calcium levels) are based on a single measurement. Biological and analytical variation may non-differentially obscure any true, underlying associations. This might be a problem especially when analytes are known to vary to a high degree, as seems to be the case with PTH [152]. It is therefore possible that a true cause-effect relationship between PTH and breast cancer exists, but that it could not be detected in the present study (paper IV).

Cause and effect

The epidemiological methods used in this thesis make it possible to test whether biologically founded hypotheses regarding causality are corroborated by statistically significant associations. As has been discussed, there are several biological explanations for the observed findings. However, it truly needs to be emphasised that it is impossible to make any inferences as to causality on the basis of the statistical associations in this thesis. Furthermore, some of the present work might be characterised as hypothesis-generating or exploratory, specifically paper III, and the results, even when statistically significant, need to be confirmed in other clinical and experimental studies.

Chance findings and statistical power

The results obtained by the statistical tests used in this thesis are all uncertain to some extent. The precision of tests is stated, for example in terms of *p*-values and confidence intervals, CI. The more tests that are performed, the higher is the risk that some observed statistical associations are due to chance alone. For instance, the positive association between serum calci-

um and breast cancer risk in premenopausal women in paper IV should be regarded with some caution since it was based on a small number of cases, the CI was broad, there was no clear dose-relationship and, indeed, results were in conflict with those obtained in paper I. Another example is paper III, where several subgroup analyses were performed, with few cases in each group, and correspondingly some CIs were wide.

However, when statistical associations are strong, i.e. with narrow CIs and distinct dose-response-relationships, and there are biologically sound explanations for a cause-effect relationship, it is reasonable to assume that observed associations are real. For example, results were consistent and showed a clear dose-response relationship regarding the positive association between calcium and breast cancer incidence and aggressiveness in overweight women (paper III and IV), suggesting a non-random finding.

Another issue is statistical power, i.e. the potential of the tests to detect any true, underlying associations. The larger the number of individuals (and cases), the more accurate the statistical predictions become. If real associations are weak, a larger number of observations are needed to detect them. When associations between exposure and outcome are inverse in different groups, for instance the relationship between serum calcium in pre- vs. peri/postmenopausal women, it makes sense to stratify the cohort according to this characteristic. Stratifying, however, splits the cohorts into smaller groups, which decreases statistical power. Hence, real associations might not be detected, due to statistical uncertainty. For example, the hypothesis that vitamin D protects against breast cancer is supported by both clinical and experimental data. However, the association between 25OHD levels and breast cancer was not statistically significant in either the present study (paper IV) or in three previous prospective case-control studies [15–17]. Gathering more observations, for example by pooling data from different studies, might achieve the needed statistical power.

Reverse causality

If breast cancer itself causes alteration of calcium, vitamin D and PTH levels, for example due to production of pHPT as in hypercalcemia of malignancy, this could lead to a spurious association between levels of these factors and breast cancer risk. In this thesis, subjects with prevalent breast cancer at baseline were excluded from study groups.

Hypercalcemia of malignancy usually presents with advanced disease [86], and such patients would probably have been diagnosed with breast cancer at baseline and would, thus have been excluded. Furthermore, additional analyses excluding cases diagnosed within two years of baseline inclusion from the analyses in order to exclude cases with subclinical disease, did not substantially change results. Hence, it is unlikely that reverse causality plays any role in the findings of the present thesis.

Implications and future studies

The findings that serum calcium is positively associated with breast cancer incidence and aggressiveness (papers I, III and IV) is in line with previous epidemiological studies that found positive associations with calcium levels and increased mortality [114]. However, some experimental studies on calcium and breast cancer indicate that calcium has tumour protective effects *in vitro* [85, 142]. Thus, the findings of this thesis concerning serum calcium and breast cancer must be confirmed in future studies. However, the results suggest that calcium might be causally related to breast cancer induction and/or growth.

The present results for 25OHD and breast cancer risk were similar to those reported by others [15–17], and the results for calcium and breast cancer risk in overweight women

were similar in the two cohorts included in the current thesis.

The lack of any association between PTH and breast cancer risk might have several explanations; there may indeed be no true association, but it may also be that the large biovariability in PTH levels [151, 152] attenuates any true associations; or that PTH only has a tumour promoting effect above a certain level, i.e. a threshold effect.

Conclusions

- Serum calcium is positively associated with breast cancer risk in overweight and peri-/postmenopausal women (paper I and IV). Serum calcium seems to be associated with breast cancer risk in premenopausal women. The exact relationship remains to be determined. One study showed a negative association (paper I), whereas another study showed a positive association (paper IV).
- Premenopausal status, use of oral contraceptives and hormone-replacement therapy, are negatively associated with serum calcium levels. BMI is significantly associated with serum calcium levels, with lean and overweight women having higher calcium levels than women with BMI between 20 and 25. Season is also associated with serum calcium levels, with higher levels during spring and autumn (paper II).
- Pre-diagnostic serum calcium is positively associated with increased tumour aggressiveness (the degree of cellular atypia, rate of proliferation and propensity to metastasise) in premenopausal and/or overweight women (paper III).
- There may be a weak, inverse association between 25OHD and breast cancer, but this association was not statistically significant. There was no association between PTH and breast cancer risk (paper IV).

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Populärvetenskaplig sammanfattning på svenska

Bröstcancer är den vanligaste cancerformen hos kvinnor – årligen drabbas ungefär 7 000 svenska kvinnor av sjukdomen. Trots mångårig intensiv forskning är orsakerna till sjukdomen ofullständigt kända. Ett stigande antal rapporter antyder att kalcium och dess reglerande hormoner, dvs. bisköldkörtelhormon (PTH) och vitamin D påverkar risken att få bröstcancer. Denna avhandling består av fyra delarbeten, som vart och ett har undersökt olika aspekter av detta potentiella samband.

I första delarbetet studerades risken att få bröstcancer beroende på kalciumnivå i blodprov tagna före insjuknandet. Sammanlagt nästan 8 000 kvinnor ingick i studien, och resultaten visade att hos överviktiga kvinnor efter klimakteriet ökade risken för bröstcancer med ökande kalciumnivåer i blodet. Hos kvinnor före klimakteriet var förhållandet omvänt – ökande kalciumnivåer var förknippade med lägre risk för bröstcancer.

I arbete två undersöktes samvariationen mellan kalciumnivåer och kända riskfaktorer för bröstcancer. Kalciumnivåerna var lägre hos premenopausala kvinnor (före klimakteriet), hos dem som tog p-piller eller hormonersättning, hos de yngsta och hos de äldsta samt hos dem med body mass index (BMI) mellan 20 och 25 jämfört med dem med BMI mindre än 20 och dem med BMI högre än 25.

I det tredje arbetet studerades om kalciumnivåer var relaterade till ökad risk av mer aggressiva brösttumörer, dvs. sådana med spridning till lymfkörtlar eller med en mikroskopiskt mer elakartad bild. Hos kvinnor före klimakteriet respektive hos överviktiga kvinnor var högre kalciumnivåer associerade med mer aggressiv bröstcancer.

I det fjärde arbetet undersöktes hur vitamin D-nivåer, bisköldkörtelhormonnivåer och kalciumnivåer tillsammans och var för sig påverkar bröstcancerrisken. Resultaten antyder att det är möjligt att högre vitamin D-nivåer är förknippade med en lägre bröstcancerrisk, men detta är inte statistiskt säkerställt. Däremot var högre kalciumnivåer återigen statistiskt säkerställt relaterade till en ökad risk för bröstcancer hos överviktiga kvinnor. I motsats till resultaten i första delarbetet talade analysen i arbete fyra för att premenopausala kvinnor också har en ökad bröstcancerrisk med ökande kalciumnivåer i blodet. Den statistiska styrkan av detta samband var i arbete fyra något osäkrare och fyndet måste bekräftas i andra, större studier.

Sammantaget talar dessa fyra arbeten för att kvinnor med BMI högre än 25 verkligen har en ökad risk att insjukna i bröstcancer med ökande kalciumnivåer i blodet, och att denna riskökning framför allt gäller mer aggressiv sjukdom. Avhandlingens styrka är bl.a. det stora antalet kvinnor som deltagit i studien samt det förhållandevis stora antalet cancerfall, vilket gör de statistiska analyserna överlag säkra. Ytterligare fördelar är kvalitén hos de analysmetoder som använts.

En svaghet är att kalcium, vitamin D och bisköldkörtelhormonvärdena bara mättes vid ett tillfälle och att naturligt förekommande variationer kan ha maskerat samband mellan dessa värden och bröstcancerrisk.

Fynden, att kalciumnivåer i blod hos kvinnor med BMI högre än 25 är relaterat till bröstcancerrisk och bröstcanceraggressivitet, kan emellertid, om de bekräftas av andra forskargrupper, bidra till ökad förståelse för bröstcancers uppkomst och på sikt också ge möjligheter till bättre förebyggande åtgärder och behandling.

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Paper I

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EPIDEMIOLOGY

Serum calcium and breast cancer risk: results from a prospective cohort study of 7,847 women

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Abstract Experimental and epidemiological studies suggest that calcium-regulating hormones—parathyroid hormone (PTH) and vitamin D—may be associated with breast cancer risk. No prospective cohort study has investigated the association between pre-diagnostic calcium levels and subsequent risk of breast cancer. We have examined this in a cohort of 7,847 women where serum calcium levels and established risk factors for breast cancer had been assessed at baseline. During a mean follow-up of 17.8 years, 437 incident breast cancer cases were diagnosed. Incidence of breast cancer was calculated in different quartiles of serum calcium levels and a Cox's proportional hazards analysis was used to obtain corresponding relative risks (RR), with a 95% confidence interval (CI), adjusted for potential confounders. In premenopausal women, serum calcium levels were inversely associated with breast cancer risk in a dose-response manner. The adjusted RR (95% CI) of breast cancer was in the 2nd calcium quartile 0.91 (0.65–1.30), in the 3rd quartile 0.89 (0.60–1.31), and in the 4th quartile 0.56 (0.32–0.98), as compared to the 1st calcium quartile. In postmenopausal overweight women (BMI > 25), breast cancer risk was higher in calcium quartiles 2–4 as compared to the 1st quartile. Our findings may have implications for primary prevention of breast cancer and for the management of asymptomatic primary hyperparathyroidism.

Keywords Breast cancer · Calcium · Obesity · Vitamin-D · Parathyroid hormone

Abbreviations

PTH	Parathyroid hormone
pHPT	Primary hyperparathyroidism
BMI	Body mass index
MPP	Malmö Preventive Project
HRT	Hormone replacement therapy
SD	Standard deviation
RR	Relative risk
CI	Confidence interval

Introduction

Breast cancer is the most common malignant disease in women. Most established risk factors concern reproductive history, but other factors may be of interest. Experimental studies indicate that calcium levels may affect tumor development [1–4]. The calcium-regulating hormones vitamin-D and its metabolites, most notably 1,25 $(OH)_2$ D3, and parathyroid hormone (PTH) have also been suggested to affect breast cancer risk [5, 6].

PTH and vitamin-D regulate the production of each other, and both factors increase serum calcium levels [7]. Experimental studies suggest that PTH has carcinogenic and tumor promoting effects [8–10]. At least three record-linkage studies have found a weak positive correlation

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between risk of breast cancer and primary hyperparathyroidism (pHPT), a condition with high PTH and often high calcium levels [11–13]. Contrary to this, it has been suggested that high vitamin-D levels may have tumor protective effects [6, 14]. Thus, the serum calcium level, either in itself or as a marker of certain conditions, may be associated with breast cancer risk. This relation is complicated, however, by the fact that potential mechanisms act in opposite directions.

Furthermore, several conditions may modify the relation between the factors mentioned above and the risk of breast cancer. High age and obesity are associated with a high prevalence of pHPT [15, 16] and low levels of vitamin-D, e.g., 25(OH)D [17]. It has also been suggested that pre- and postmenopausal women may have different risk factors for breast cancer [18] and that obesity may modify the association between established risk factors and breast cancer [19].

To our knowledge, there has been no prospective cohort study on serum calcium levels in relation to incidence of breast cancer. Here we report a cohort of 7,847 women, with information on total serum calcium and risk factors for breast cancer, followed with regard to breast cancer incidence during an average of 17.8 years.

The aim of the present analysis was to study incidence of breast cancer in women according to pre-diagnostic levels of serum calcium, with special regard to body mass index (BMI) and menopausal status.

Materials and methods

The Malmö Preventive Project

The Malmö Preventive Project (MPP) in Malmö, Sweden, was established in 1974 for screening with regard to cardiovascular risk factors [20]. Entire birth-year cohorts, men and women, registered as citizens in Malmö were successively invited by mail to a health examination. Approximately 70% of invited women attended [20]. When the department closed in 1992, 10,902 women, born between 1926 and 1949, had been examined. Their mean age at baseline was 49.7 years, and 59.8% were peri-/postmenopausal [21].

Baseline examination

A self-administered questionnaire was used for a comprehensive interview on lifestyle habits, medical history, marital status, education, and use of medications [20]. The questionnaire was revised several times. Information on reproductive history was included in the questionnaire for women screened from April 1983 and onwards. Factors that have been associated with breast cancer risk were available from the questionnaire: age at menarche, menopausal status, parity, use of oral contraceptives and hormonal replacement therapy (HRT), educational level, and marital status. Information necessary for calculation of body mass index (BMI) (kg/m^2) was assessed by a trained nurse on baseline examination; height was measured to the nearest centimeter, and weight was recorded with the subject wearing no shoes, on a beam scale at intervals of 0.1 kg. In the morning, after an overnight fast, all subjects gave a blood sample, which was analyzed immediately. Calcium was measured photometrically by the laboratory at the Dept. of Clinical Chemistry, University Hospital of Malmö, on a PRISMA multichannel autoanalyser (Clinicon AB, Bromma, Sweden) [22, 23]. The coefficient of variation was 1.52% [22]. The reference value for serum calcium during this period was 2.20–2.60 mmol/l. Women were classified as peri-postmenopausal if they affirmed that their menstruations had ceased, that they had menopausal symptoms or that they were taking any ''female hormonal medication'' because of such symptoms.

Study cohort

Out of 10,902 women, information on reproductive factors including menopausal status was known for 8,051 women. Serum calcium had been measured in 8,004 of these. A total of 157 women with prevalent invasive breast cancer at baseline were excluded. Thus, the present study is based on 7,847 women.

Ethical clearance for this study was obtained from the Ethical committee in Lund, LU 639-03.

Follow-up

Breast cancer cases, invasive and in situ, were retrieved by record linkage with the Swedish Cancer Registry and the Southern Swedish Regional Cancer Registry. There were in all 437 incident cases. Stage at diagnosis was retrieved from clinical notes and from a clinical registry run by the South Swedish Breast Cancer Group. Stage according to the UICC was given from the TNM classification [24]. Information on vital status was retrieved from the Swedish Cause of Death Registry. Each woman was followed until end of follow-up, 31st December 2003, or until she got breast cancer or died. The average follow-up was 17.8 years (SD: 5.8 years).

Statistical methods

Quartile cut-points for total serum calcium were based on the distribution for women in the study cohort excluding those with prevalent cancer of any site (not including

cervical cancer in situ). The incidence of breast cancer per 100,000 person-years was calculated in relation to serum calcium quartiles. Cox's proportional hazards analysis was used to estimate corresponding relative risks (RRs), with a confidence interval (CI) of 95%. In a second analysis, potential confounders were introduced as covariates. BMI and age were treated as continuous variables, whereas all other covariates were entered as categorical variables. Test for trend over quartiles were calculated and a p-value < 0.05 was considered statistically significant.

Separate analyses were made in pre- versus peri-/post-menopausal women and in different strata of BMI, i.e., BMI < 25 vs. BMI ≥ 25 (overweight and obese women). All analyses were repeated excluding cases diagnosed within two years following baseline examination. Analyses were also done using separate quartile cut-points based on serum calcium levels in pre- versus peri-postmenopausal women.

Stage distribution was assessed in relation to serum calcium quartiles, BMI, and postmenopausal status. The chi-square test was used in order to assess heterogeneity between groups, and a p-value less than 0.05 was considered statistically significant.

Results

Use of oral contraceptives and hormone replacement therapy, HRT, were more common in lower calcium quartiles, and there was a higher percentage of peri-post-menopausal women in higher calcium quartiles, Table 1. Mean calcium levels (range) were in premenopausal women 2.32 (1.89–2.76) mmol/l and in peri-postmenopausal women 2.36 (2.03–2.80) mmol/l. This is in line with previous studies that have shown serum calcium to rise with menopause [25, 26].

Table 1 Distribution of potential risk factors for breast cancer according to serum calcium level

Factor[a]	Serum calcium quartile [mmol/l]				
	1 n = 1,880 [<2.29]	2 n = 2,109 [2.29–2.34]	3 n = 2,034 [2.35–2.40]	4 n = 1,824 [>2.40]	All n = 7,847
	(column percent; *mean (SD) in italics*)				
Age at baseline (years)	*51.4 (4.5)*	*52.0 (4.6)*	*52.5 (4.5)*	*53.5 (4.0)*	*52.3 (4.5)*
BMI (kg/m^2)	*24.7 (4.2)*	*24.6 (4.2)*	*24.9 (4.2)*	*25.1 (4.4)*	*24.8 (4.2)*
<12 years at menarche(vs. ≥12 years)	11.8	13.1	12.0	13.7	12.6
Oral contraception (yes versus no)	8.4	5.3	3.8	2.0	4.9
Peri-/postmenopausal (versus premenopausal)	46.6	59.4	69.6	78.8	63.5
Number of children					
0	13.9	14.6	17.4	17.2	15.8
1–2	59.5	59.6	56.6	56.4	58.0
3–4	25.2	24.5	24.3	24.8	24.7
>4	0.9	1.1	1.0	1.2	1.1
HRT among peri-/postmenopausal (n = 4980) (vs. no-use)	24.7	16.0	10.1	8.3	13.6
Smoking status					
Never	47.7	46.6	46.5	49.0	47.4
Ex	20.5	20.7	19.2	18.7	19.8
Current	31.9	32.7	34.3	32.3	32.8
Married					
Yes	51.2	4.8.7	50.6	50.3	50.2
No	44.9	44.9	42.1	41.2	43.3
Missing	3.9	6.4	7.3	8.6	6.5
Alcohol consumption					
None	15.6	15.3	16.9	18.1	16.5
Less than every week	59.4	61.0	58.8	58.7	59.5
Every week	25.0	23.7	24.3	23.2	24.1
≥12 years of education	34.4	32.4	32.1	32.0	32.7

[a] Separate missing category reported if > 1% of subjects had no information

Table 2 Breast cancer incidence in pre-, peri/postmenopausal and all women in relation to serum calcium levels

Menopausal status	Serum calcium quartile	Individuals	Breast cancer cases	Person-years	Incidence/ 100,000	RR (CI: 95%)	RR[a] (CI: 95%)
Premenopausal	1	1,003	72	17,281	417	1.00	1.00
	2	857	56	14,763	379	0.91 (0.65–1.30)	0.92 (0.65–1.31)
	3	618	39	10,626	367	0.89 (0.60–1.31)	0.88 (0.59–1.30)
	4	386	15	6,518	230	0.56 (0.32–0.98)	0.56 (0.32–0.99)
Peri/postmenopausal	1	877	39	14,055	277	1.00	1.00
	2	1,252	66	20,335	324	1.17 (0.79–1.74)	1.20 (0.81–1.79)
	3	1,416	80	22,185	361	1.31 (0.89–1.92)	1.38 (0.93–2.03)
	4	1,438	70	21,899	320	1.17 (0.79–1.73)	1.26 (0.84–1.89)
All	1	1,880	111	31,336	354	1.00	1.00
	2	1,987	122	35,098	348	0.98 (0.76–1.27)	0.99 (0.76–1.28)
	3	1,915	119	32,811	363	1.03 (0.80–1.34)	1.05 (0.81–1.36)
	4	1,739	85	28,417	299	0.86 (0.65–1.14)	0.89 (0.67–1.19)
Total		7,847	437	127,662	342		

[a] adjusted for age, BMI, age at menarche, use of oral contraception, number of children, use of hormone-replacement therapy (in peri-/postmenopausal women), smoking status, marital status, alcohol consumption and educational level

There was no overall association between serum calcium levels and breast cancer, Table 2, crude and age-adjusted RR:s were similar to those obtained in the full model.

Serum calcium levels were inversely associated with incidence of breast cancer in premenopausal women in a dose-response manner, but p for trend did not reach statistical significance, $p = 0.25$. In postmenopausal women, the p-value for trend over quartiles was 0.45. There was a weak, non-significant, association between serum calcium levels and breast cancer incidence in peri-/postmenopausal women.

Serum calcium was associated with a high risk of breast cancer in overweight postmenopausal women, Table 3. The RR:s, adjusted for known risk factors for breast cancer, as outlined in Table 1, for the 2nd, 3rd and 4th quartiles as compared to the 1st were 2.74, 3.10 and 2.72, respectively (p for trend: 0.04). Crude and age-adjusted RR:s were similar. There was no statistically significant trend over quartiles in any of the analyses presented in Table 3. In the subgroup of obese, postmenopausal women the distribution of other risk factors was similar in all calcium quartiles.

When the analyses were repeated excluding cases with breast cancer occurring within 2 years following baseline,

Table 3 Breast cancer incidence in pre- and peri/postmenopausal women in relation to serum calcium in different strata of bmi

Menopausal status	Serum calcium quartile	BMI < 25			BMI ≥ 25		
		Individuals	Cases	RR[a] (CI: 95%)	Individuals	Cases	RR[a] (CI: 95%)
Premenopausal	1	650	45	1.00	353	27	1.00
	2	582	35	0.91 (0.58–1.43)	275	21	0.98 (0.55–1.76)
	3	405	28	1.04 (0.65–1.69)	213	11	0.60 (030–1.22)
	4	228	10	0.68 (0.34–1.35)	158	5	0.44 (0.16–1.15)
Peri/postmeno-pausal	1	518	31	1.00	359	8	1.00
	2	736	35	0.82 (0.50–1.34)	516	31	2.74 (1.25–5.98)
	3	797	40	0.91 (0.57–1.47)	619	40	3.10 (1.44–6.68)
	4	795	37	0.88 (0.54–1.44)	640	33	2.72 (1.24–5.94)
All	1	1168	76	1.00	712	35	1.00
	2	1318	70	0.84 (0.61–1.17)	791	52	1.34 (0.87–2.06)
	3	1202	68	0.95 (0.68–1.32)	832	51	1.26 (0.81–1.94)
	4	1023	47	0.82 (0.56–1.19)	798	38	1.09 (0.68–1.74)
Total		4711	261		3133	176	

[a] adjusted for age, age at menarche, use of oral contraception, number of children, use of hormone-replacement therapy (in peri-/postmenopausal women), smoking status, marital status, alcohol consumption and educational level

all results were similar (data not shown). Results were also similar when using separate quartile cut-points based on serum calcium levels in pre- versus postmenopausal women (data not shown); however, results did not reach statistical significance in the 4th calcium quartile among obese peri-postmenopausal women, RR: 1.76 (0.92–3.38).

Stage was known for 422 out of 437 breast cancer cases (96.6%). There was no clear relation between stage distribution and serum calcium quartiles. Thus, 31.5% of cases in the 1st calcium quartile were diagnosed with a stage II + tumor (stage II, III or IV), 37.4% in the 2nd, 29.5% in the 3rd, and 43.0% in the 4th serum calcium quartile. These differences corresponded to a *p*-value of 0.21. Stage distribution in different serum calcium quartiles was also similar in pre- versus. postmenopausal and in normal versus overweight/obese women.

Discussion

In this study serum calcium levels were inversely associated with breast cancer risk in premenopausal women in a dose-response manner. This study also indicates that calcium levels are positively associated with breast cancer in overweight peri-/postmenopausal women.

It may be questioned whether it is appropriate to use a single determination for ranking of serum calcium levels. Both short-time [27] and long-time [28] intra-individual variation in total serum calcium are low. Even though serum calcium levels rise with menopause [26, 29] there seems to be significant 'tracking', i.e., the ranking of calcium levels between individuals tends to remain the same before and after menopause [25]. Although inter-individual differences in absolute values for serum calcium are low, we still consider these differences important when large groups are compared. Thus, we believe that a single measurement of serum calcium is a useful marker for differences with regard to calcium homeostasis.

It has been argued that free (ionized) calcium provides the best indication of calcium status because it is biologically active and tightly regulated by calcium-regulating hormones. Total calcium levels are affected by plasma protein levels, notably albumin. In our cohort, adjusting for serum albumin was not possible, since albumin levels were only known for about a quarter ($n = 2048$) of the study population. However, total calcium has been considered a good measure of calcium homeostasis in outpatients and healthy individuals where albumin will be expected to be in the normal range [23]. Albumin was normally distributed among those with known albumin levels, and only 75 women (3.7%) had an albumin outside the normal reference range (36–45 g/l). All samples were also collected in a standardized manner, which minimizes differences in albumin levels due to fasting status or diurnal variation [30]. Following this, we consider total serum calcium a useful and valid measurement of calcium status in this study population.

Vitamin D and PTH-levels seem to be unaffected by menopause per se [25]. The rise of serum calcium with menopause might instead be explained by the fact that bone seems to turn more sensitive to PTH in the absence of estrogen [31, 32]. The associations between some risk factors and breast cancer differ in pre- and postmenopausal women and this was one reason a priori to study breast cancer incidence separately in pre- versus peri-postmenopausal women [33]. Moreover, pHPT is more common in postmenopausal than in premenopausal women [15]. PHPT is often a mild disease with a protracted course, asymptomatic in its early stages, with gradually rising calcium levels. The prevalence of clinical symptomatic pHPT in postmenopausal women has been estimated to be around 3% [34] and the prevalence of asymptomatic pHPT could be even higher. Whether this prevalence is high enough to affect calcium levels overall in this group might be questioned, but we think it is reasonable to assume a higher percentage of women with undiagnosed, asymptomatic pHPT in the higher serum calcium quartiles.

If breast cancer itself causes hypercalcemia, such as in hypercalcemia of malignancy, this could lead to a spurious association between calcium levels and breast cancer risk. This would be a serious problem in cross-sectional studies, i.e., case–control studies, but it is less of a problem when pre-diagnostic calcium levels are available, as in this analysis. Moreover, hypercalcemia of malignancy usually presents with advanced disease [35], and such patients would probably have been diagnosed with breast cancer at baseline and would, thus, have been excluded. To further exclude cases where hypercalcemia might have been caused by undiagnosed breast cancer, the analyses were repeated excluding cases with breast cancer occurring within two years following baseline. All results were similar and we do not consider that malignancy-related hypercalcemia associated with breast cancer have affected the observations in the present study. Malignancies other than breast cancer can also give rise to hypercalcemia. In these cases, an association between serum calcium and breast cancer could be due to calcium levels, or mediators of calcium homeostasis, affecting breast cancer growth. Hence, we did not exclude other prevalent malignancies from our analysis, but only those with prevalent breast cancer.

Incomplete follow-up may affect the results. However, the Swedish Cancer Registry and the Swedish Cause of Death Registry have been validated and found to have a completeness of about 99% [36].

Another relevant issue is whether the results could have been caused by detection bias. Women in these birth cohorts have regularly been invited to mammographic screening since the end of the 1970s [37]. If calcium levels were associated with factors that affect time of diagnosis, such as participation in mammographic screening or patient's delay, a trend over quartiles with respect to stage at diagnosis would be expected. No such trend was observed and we consider it unlikely that detection bias has influenced our results.

It may be asked whether breast cancer cases in this cohort may be considered representative of the whole breast cancer population. This cohort mainly comprised middle-aged women and 30% of the women invited to the health examination did not attend. As we have no information about exposure to the studied risk factors in women outside this cohort, observed incidence rates may not be applicable to all age groups or to the general population. However, as there was a wide distribution of calcium levels, it was possible to make internal comparisons between subjects with low and high values, respectively. We consider that our estimations of relative risks were not considerably affected by selection bias.

It is possible that both high serum calcium levels and breast cancer are caused by a common factor. The results were probably not confounded by most known risk factors for breast cancer though, since information on these were known for the study cohort, and results were similar when statistical analyses were repeatedly adjusted for these factors. We were not able to adjust our estimates for heredity or physical activity, since we had no information on these variables. It is, however, unlikely that these factors have influenced the results, since, to our knowledge, there is no strong correlation between either physical activity or heredity and serum calcium levels. Other factors that would have been of interest are determinants of calcium homeostasis, such as diet, sunshine exposure, vitamin D, and PTH-levels. The inclusion of these factors in future studies would be very valuable.

This is the first prospective cohort study on serum calcium levels and breast cancer risk. In order to explore whether factors that affect PTH and vitamin-D levels, i.e., menopausal status and obesity, modify the relation between calcium levels and breast cancer risk, several subgroup analyses were performed. Some groups had a limited number of cases and our finding that high calcium levels are associated with breast cancer in overweight peri-/postmenopausal women was based on a low number of cases and CIs were wide. However, the distribution of risk factors in postmenopausal obese women between calcium quartiles does not differ from the whole study population (data not shown) and the results may represent a true threshold-effect in RR between the first and second calcium quartiles. Indeed, it may be that the lowest quartile has a lower than average risk; that low calcium levels in obese postmenopausal women reflects some protective factor. Such factors may be related to parameters that affect calcium homeostasis, i.e., PTH or vitamin-D, but this remains to be evaluated. Given the small number of cases in some subgroups, a chance finding cannot completely be ruled out, and our results will have to be confirmed in larger studies.

High levels of calcium per se can in experimental models increase cell differentiation, decrease proliferation, and induce apoptosis [1–4]. All of this would have a tumor protective effect. Calcium levels in serum may also be considered a marker for certain conditions, as calcium levels are increased following stimulation by PTH and vitamin-D. Experimental studies have shown that PTH have anti-apoptotic effects and may promote invasiveness [8–10], mechanisms that stimulate tumor growth. Contrary to this, vitamin-D may induce apoptosis, cell cycle arrest, and differentiation. Vitamin-D also inhibits invasiveness and angiogenesis [6, 14, 38], all of which have tumor protective effects. Following these potential biological mechanisms, it is possible to hypothesize that high serum calcium levels may be associated with both tumor protective and tumor promoting effects. To date, no study has investigated the influence of calcium levels as well as PTH and vitamin-D levels on breast cancer risk.

There is typically a weak, non-significant, inverse association between calcium intake and risk of breast cancer as reported by at least eight case–control studies and three cohort studies (referred to in [39, 40]). However, calcium homeostasis is kept very tight in humans, and dietary intake is a poor predictor of calcium levels in blood. No prospective cohort study has investigated the association between serum calcium levels in blood and breast cancer incidence. Three epidemiological record-linkage studies have evaluated primary hyperparathyroidism (pHPT) and risk of breast cancer [11–13]. Two of these studies found a statistically significant positive association between pHPT and subsequent breast cancer.

Two studies have examined vitamin-D levels in pre-diagnostic blood samples in relation to breast cancer risk. Hiatt et al. did not find any significant association between the vitamin-D metabolite $1,25(OH)_2D$ and breast cancer, but included only 96 cases [41]. Bertone-Johnson et al. found that the vitamin-D metabolites 25(OH)D and $1,25(OH)_2D$ were associated with a small, non-significantly, decreased risk of breast cancer [42]. None of these studies included information on calcium levels or PTH.

Studies on the relation between dietary intake of vitamin-D, or dairy products, and risk of breast cancer, do not, however, provide consistent evidence for an association [4, 40, 42, 43].

High calcium levels may reflect different conditions in specific groups as pHPT and low vitamin-D-levels are more common in postmenopausal [15, 17] and obese women [16]. It is possible to hypothesize that calcium levels in postmenopausal and obese women may reflect PTH levels rather than vitamin-D levels and that calcium levels in premenopausal women may mainly be a marker of vitamin-D levels. Obese are known to have an altered endocrine metabolism [44] and possibly factors as insulin or insulin-like growth factor (IGF) could be related to our observations [45].

Our findings will have to be tested in future studies, which must include information on PTH and vitamin-D levels as well as on serum calcium. Further research will give guidance on primary prevention for breast cancer, and can be important when deciding whether or not to treat individuals with mild or asymptomatic hyperparathyroidism.

We conclude that in this cohort of 7,847 women, serum calcium levels were inversely associated with breast cancer risk in premenopausal women in a dose-response manner. This study also indicates that high calcium levels may increase breast cancer risk in overweight peri-/postmenopausal women.

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Paper II

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ORIGINAL ARTICLE

Reproductive history, lifestyle factors and season as determinants for serum calcium concentrations in women

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Objective. Serum calcium concentrations have been associated with the risk of malignant disease, especially breast cancer. Thus, determinants of serum calcium concentrations, with special regard to risk factors of breast cancer, are of great interest. ***Material and methods.*** Previous studies have either been small or they have not focused on reproductive factors. The present study examined serum calcium concentrations in relation to reproductive history, selected lifestyle factors and screening season in a large population-based cohort study comprising 8,114 women. ANOVA followed by the Bonferroni *t*-test were used for comparison of means, and logistic regression and multiple regression analysis were used to test associations. ***Results.*** Serum calcium concentrations were lower in hormone replacement therapy users versus non-users (2.321 mmol/L versus 2.364; $p<0.001$) and in users of oral contraceptives versus non-users (2.304 versus 2.348; $p<0.001$). They were higher in peri-/postmenopausal versus premenopausal women (2.357 versus 2.319; $p<0.001$). Overweight and obese women had higher mean calcium concentrations (2.350 and 2.355) than women with body mass index between 20 and 25 (2.342; $p<0.001$). Serum calcium concentrations were higher in spring and autumn than in winter (2.352 and 2.353 versus 2.343; $p=0.002$). Both younger (40–45 years) (2.334; $p=0.001$) and older age groups (>55 years) (2.363; $p<0.001$) had higher mean calcium concentrations compared to those of women aged 45–50 years (2.320), even when adjusting for menopausal status, suggesting that age has an independent influence on calcium concentrations. ***Conclusions.*** It is concluded that reproductive factors such as menopausal status, use of oral contraceptives or hormone-replacement therapy, and age, BMI and season are associated with serum calcium concentrations.

Keywords: Body mass index; calcium; oestrogens; parathyroid hormone; vitamin D

Introduction

Serum calcium concentrations have been associated with the risk of both cardiovascular [1] and malignant disease, especially breast cancer [2,3]. Thus, determinants of serum calcium concentrations are of great interest. Since reproductive factors are important risk factors for breast cancer, it is particularly important to study the association between these factors and serum calcium concentrations.

Serum calcium is tightly regulated by vitamin D and parathyroid hormone (PTH) and may be affected by a number of important diseases, such as osteoporosis, obesity and malignancy. Moreover, both vitamin D and PTH have been suggested to influence breast cancer risk [4,5]. Previous studies have found serum calcium concentrations to be associated with reproductive factors such as menopausal status [6], oestrogen concentrations [7], phase of the menstrual cycle [8,9] and pregnancy [10]. These studies have been well designed, but were relatively small, including at most fewer than a few hundred subjects, and selected from specific groups of patients. In a relatively large cohort study including 519 patients, significant associations were also found between reproductive factors and serum calcium [11]. The largest study to date, the cohort study reported by Jorde et al., did not focus on reproductive factors, but discussed other potential risk factors related to lifestyle, such as body mass, smoking habits and alcohol consumption [1]. Concentrations of vitamin D have a distinctive seasonal variation [12], but it is not clear whether serum calcium varies over the year [13]. There is therefore need for a large population-based cohort study investigating the impact of reproductive history, lifestyle factors and season on serum calcium concentrations.

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The aim of our study was to examine serum calcium concentrations in relation to reproductive history, selected lifestyle factors and screening season in a large population-based cohort study comprising 8,114 women.

Material and methods

The Malmö Preventive Project

The Malmö Preventive Project (MPP) in Malmö, Sweden, was established in 1974 for screening in regard to cardiovascular risk factors [14]. Entire birth-year cohorts, men and women, registered as citizens in Malmö were successively invited by mail to take a health examination. Approximately 70 % of invited women attended [14]. When the department closed in 1992, 10,902 women, born between 1926 and 1949, had been examined. Their mean age at baseline was 49.7 years and 59.8 % were peri-/postmenopausal [15].

Baseline examination

A self-administered questionnaire was used for a comprehensive interview on lifestyle habits such as obesity, smoking and alcohol consumption. Information necessary for calculation of body mass index (BMI) (kg/m^2) was assessed by a trained nurse at baseline examination; height was measured to the nearest centimeter and weight was recorded on a beam scale at intervals of 0.1 kg. Women were classified as peri- or postmenopausal if: they affirmed that their menstruation periods had ceased, that they had menopausal symptoms or that they were taking any ''female hormonal medication'' due to such symptoms. The question ''Have you ever smoked daily for at least 6 months?'' was used to define never and ever smokers. Ever smokers who did not confirm that they were still smoking were considered ex-smokers. Alcohol consumption was divided into three categories (low, medium and high) using a modified version of MAST (the Michigan Alcoholism Screening Test), as previously described [16].

The questionnaire was revised several times. Information on reproductive history was included in the questionnaire for women screened from April 1983 and onwards. Reproductive factors available from the questionnaire were: age at menarche, menopausal status, parity, use of oral contraceptives and hormonal replacement therapy (HRT).

There were few women examined in June, July and August. Season was defined as follows: summer=May to August; autumn=September and October; winter=November to February; and spring=March and April.

The cohort was linked to the Swedish Cancer Registry in order to obtain information on prevalent cancer at baseline [2]. The current study did not include previous cancer *in situ* of the uterine cervix in the definition of prevalent cancer at baseline.

Serum calcium measurements

In the morning, after an overnight fast, all subjects gave a blood sample which was analysed immediately. Calcium was measured photometrically by the laboratory at the Department of Clinical Chemistry, University Hospital of Malmö, on a PRISMA multichannel autoanalyser (Clinicon AB, Bromma, Sweden) [17,18]. The coefficient of variation (CV) was 1.52 % [18] and the reference value for adult women for serum calcium during this period was 2.20–2.60 mmol/L.

Study cohort

Out of 10,902 women in the Malmö Preventive Project, 8,161 women were examined following April 1983, i.e. when questions on reproductive factors were included. Out of these women, 8,114 had information on serum calcium concentrations. At inclusion, women were aged 42 to 58 years, with a median age of 54 years. Ethical clearance for the study was obtained from the Ethics Committee in Lund, LU 639-03.

Statistical methods

The study cohort was divided into sixtiles based on serum calcium concentrations at baseline. The distribution of reproductive and selected lifestyle factors was investigated in relation to serum calcium concentrations. Means of serum calcium were calculated in different categories of the studied factors. Confidence intervals were chosen as an estimation of the accuracy of the estimated means. In order to test differences in mean calcium concentrations between different categories of the studied factors, ANOVA was used followed by a Bonferroni *t*-test. All tests were two-sided and a p-value <0.05 was considered statistically significant. Subjects with missing information were included as a separate category.

All women were dichotomized into low ($\leqslant$2.34 mmol/L) and high ($\geqslant$2.35 mmol/L) calcium concentrations, the median of the study cohort. Odds ratios with 95 % confidence intervals (CI) were calculated for high versus low concentrations, in relation to the studied factors, using a logistic regression analysis. The second model was adjusted for age and a final model included all studied factors.

Missing values were coded as a separate category, except for BMI, where this information was only missing for three subjects and missing values were left as blank. Calcium concentrations were approximately normally distributed and the relation between different factors and calcium concentrations was further investigated using multiple linear regression analysis. All categorical variables in the linear regression analysis were transformed and entered as multiple categorical variables. Partial regression coefficients (β_i) with 95 % confidence intervals, adjusted for all other factors, were reported. A stepwise multiple regression was undertaken, with *p*-values for inclusion set at 0.05 and exclusion at 0.10. All analyses were repeated excluding subjects with prevalent cancer at baseline.

Results

Serum calcium concentrations were overall normally distributed, with 95 % of the study population having values between 2.21 and 2.49 mmol/L.

Use of oral contraceptives and hormone replacement therapy (HRT) were more common in lower calcium sixtiles, and there was a higher percentage of older and peri/postmenopausal women in higher calcium sixtiles (Table I). Mean serum calcium concentrations tended overall to be located in a narrow interval, and corresponding CIs were well within the normal reference range at the time (Table II). Mean calcium concentrations were higher in subjects aged 40–45 and $\geqslant$50 years of age compared to those of participants aged 45–50 years (Table II). Overweight and obese women had significantly higher mean calcium concentrations compared to women with a BMI between 20 and 25.

Considering reproductive factors, mean calcium concentrations were higher in peri-/postmenopausal women versus premenopausal women, and in women without hormonal therapy (i.e. HRT or OC) versus women receiving such therapy.

Calcium concentrations varied in relation to season: the highest mean calcium concentrations were found in women screened in spring and autumn.

The adjusted logistic regression analysis revealed statistically significant ORs for high versus low calcium concentrations in relation to age, BMI, OC, HRT, menopausal status and screening season (Table III). Both lean (BMI $<$ 20) and overweight/obese women (BMI $>$ 25) had a higher odds ratio for high calcium concentrations than had women with a BMI of 20 to 25. The same was found regarding age: the youngest age group (40–45 years) and the oldest (50 years and above) had a higher odds ratio for high calcium concentrations than that of women aged 45–50 years. Premenopausality, use of OC and HRT all had higher odds ratio for low serum calcium concentrations. The OR for high serum calcium concentrations was highest in those screened in spring and autumn. There was no significant correlation between smoking and serum calcium concentrations in this cohort. There was a significant positive association between alcohol consumption and calcium concentrations in the logistic regression; however, there was no association between alcohol consumption and serum calcium in the regression analysis, nor in the ANOVA.

The multiple regression analysis showed that higher age, peri/postmenopausality, non-use of HRT or OC and menarche after 12 years of age were all associated with high calcium concentrations (Table III). *R* and adjusted *R* squared were 0.297 and 0.088, respectively. In the stepwise regression analysis, the following factors remained as predictors of serum calcium: menopausality, use of HRT, use of oral contraceptives, age at screening, number of children and age at menarche. BMI, alcohol consumption and smoking status did not remain in the model. *R* in the stepwise analysis was 0.269, with adjusted *R* squared 0.072.

Results were similar in all analyses when excluding individuals with prevalent cancer at baseline (data not shown).

Discussion

In this large, population-based cohort comprising 8,114 women, high serum calcium concentrations were positively associated with menopause and age, and negatively associated with use of HRT, use of OC and menarche at 12 years of age or younger. BMI was significantly associated with serum calcium concentrations, with lean and overweight women having higher calcium concentrations than women with BMI between 20 and 25. Season was also associated with serum calcium concentrations, with high concentrations during spring and autumn.

In our study, both younger (40–45 years) and higher age groups ($>$55 years) had higher calcium concentrations compared to those of women aged 45–50 years, even when adjusting for menopausal status, suggesting that age has an independent influence on calcium concentrations. It may be that younger women have higher vitamin D concentrations than do middle-aged women, whereas older women might have higher PTH concentrations than middle-aged women as a consequence of more prevalent asymptomatic pHPT in this age group [19]. As for BMI, it is known that obese often have

Table I. Serum calcium sixtiles in relation to reproductive history, life-style factors, and season.

	Serum calcium sixtile [mmol/L]						
	1 [⩽2.26] n=1354	2 [2.27–2.30] n=1245	3 [2.31–2.34] n=1512	4 [2.35–2.38] n=1466	5 [2.39–2.42] n=1162	6 [>2.43] n=1375	All n=8114
Factor [number if n<8114]	Column percent; *mean (SD) in italics*						
Age (years)	*51.3 (4.5)*	*52.0 (4.5)*	*51.9 (4.6)*	*52.5 (4.4)*	*52.9 (4.3)*	*53.6 (3.9)*	*52.4 (4.4)*
⩾40 – <45	12.6	12.0	13.4	11.3	10.5	7.1	11.2
⩾45 – <50	26.5	19.9	18.8	14.1	13.0	10.0	17.1
⩾50 – <55	26.2	25.5	24.1	23.1	20.8	19.0	23.1
⩾55	34.6	42.7	43.8	51.4	55.7	63.9	48.6
BMI (kg/m^2) [8111]	*24.7 (4.3)*	*24.7 (4.2)*	*24.5 (4.1)*	*24.9 (4.2)*	*25.1 (4.4)*	*25.1 (4.4)*	*24.8 (4.2)*
<20	7.8	5.9	7.7	8.0	6.8	8.1	7.4
⩾20 – <25	55.2	54.6	55.2	50.6	50.6	48.5	52.5
⩾25 – <30	27.0	29.4	27.8	29.7	29.5	30.8	29.0
⩾30	10.0	10.1	9.3	11.6	13.1	12.7	11.1
Age at menarche (years) [8021]							
<12	10.7	12.8	13.5	12.4	12.0	13.3	12.5
⩾12	88.3	86.2	85.4	86.6	86.7	85.2	86.4
Oral contraception [8056]							
No	90.7	93.7	93.9	95.6	96.1	97.1	94.5
Yes	8.8	6.0	5.2	3.6	3.2	1.8	4.8
Menopausal status							
Pre	43.5	34.1	31.0	23.8	20.1	15.6	28.1
Peri/post	56.5	65.9	69.0	76.2	79.9	84.4	71.9
Number of children [8020]							
0	13.2	14.1	15.1	17.8	17.2	16.3	15.6
1–2	58.7	59.5	59.4	55.9	56.9	55.5	57.7
3–4	26.2	24.4	23.4	24.0	23.4	25.7	24.5
>4	0.7	1.4	0.9	1.0	0.9	1.2	1.0
HRT in peri/postmenopausal [5837]							
No	67.3	76.7	83.2	87.4	88.9	90.3	83.3
Yes	32.7	23.0	16.8	12.6	11.0	9.7	16.6
Smoking status [8061]							
Never	47.9	48.0	44.8	45.9	48.2	48.1	47.0
Ex	20.3	19.7	21.4	19.1	17.8	19.7	19.7
Current	31.3	32.0	32.9	34.4	33.6	31.1	32.6
Alcohol consumption [7364]							
Low	64.0	59.4	63.3	62.1	59.8	60.7	61.7
Medium	27.9	31.3	26.3	25.6	26.4	24.5	26.9
High	1.8	2.2	2.1	2.5	2.8	1.7	2.2
Prevalent cancer							
No	95.6	95.7	95.6	95.5	94.6	94.5	95.3
Yes	4.4	4.3	4.4	4.5	5.4	5.5	4.7
Screening season							
Winter	43.4	42.2	40.3	40.3	39.5	36.9	40.4
Spring	18.0	19.0	19.7	20.1	22.9	22.2	20.3
Summer	25.8	24.5	24.3	24.1	22.5	23.3	24.1
Autumn	12.8	14.4	15.6	15.4	15.1	17.7	15.2

higher PTH concentrations [20], a form of secondary hyperparathyroidism, and this might explain the higher mean calcium concentrations in the overweight and obese. The obese, on the other hand, are more likely to have vitamin D deficiency than people with normal weight [21].

Vitamin D influences serum calcium concentrations and is produced in the skin under the influence

Table II. Mean serum calcium levels in relation to reproductive history, life-style factors, and season.

Factor	No.	Mean (95 % CI)	Bonferroni t-test (*p*-value)	ANOVA (*p*-value)
Age (years)				
⩾40 – <45	908	2.334 (2.328–2.339)	0.001	
⩾45 – <50	1386	2.320 (2.316–2.324)	Ref.	
⩾50 – <55	1878	2.337 (2.333–2.341)	<0.001	
⩾55	3942	2.363 (2.360–2.365)	<0.001	<0.001
BMI (kg/m^2)				
<20	604	2.348 (2.341–2.354)	0.721	
⩾20 – <25	4256	2.342 (2.339–2.344)	Ref.	
⩾25 – <30	2354	2.350 (2.347–2.354)	0.001	
⩾30	897	2.355 (2.349–2.361)	<0.001	<0.001
Age at menarche (years)				
<12	1013	2.351 (2.346–2.356)	0.147	0.032
⩾12	7008	2.345 (2.343–2.347)	Ref.	
Oral contraception				
No	7668	2.348 (2.346–2.350)	Ref.	
Yes	388	2.304 (2.295–2.312)	<0.001	<0.001
Menopausal status				
Pre	2277	2.319 (2.316–2.323)	Ref.	
Peri/post	5837	2.357 (2.355–2.359)	<0.001	<0.001
Number of children				
0	1269	2.353 (2.349–2.358)	0.012	
1–2	4678	2.344 (2.342–2.347)	Ref.	
3–4	1990	2.346 (2.342–2.350)	1.000	
>4	83	2.347 (2.329–2.366)	1.000	0.006
HRT in peri-/postmenopausal				
No	4864	2.364 (2.362–2.366)	Ref.	
Yes	970	2.321 (2.315–2.326)	<0.001	<0.001
Smoking status				
Never	3817	2.347 (2.344–2.349)	Ref.	
Ex	1602	2.344 (2.339–2.348)	1.000	
Current	2642	2.347 (2.344–2.350)	1.000	0.142
Alcohol consumption				
Low	5003	2.345 (2.342–2.347)	Ref.	
Medium	2186	2.342 (2.339–2.346)	1.000	
High	175	2.348 (2.336–2.359)	1.000	<0.001
Prevalent cancer				
No	7729	2.346 (2.344–2.348)	Ref.	
Yes	385	2.355 (2.346–2.364)	0.034	0.034
Screening season				
Winter	3279	2.343 (2.340–2.346)	Ref.	
Spring	1644	2.352 (2.348–2.356)	0.002	
Summer	1959	2.343 (2.340–2.347)	1.000	
Autumn	1232	2.353 (2.348–2.358)	0.002	<0.001

* Compared to reference (*p*-value).

of sunlight. It is possible to hypothesize that the degree of sun exposure through increased vitamin D concentrations would lead to higher calcium concentrations in summer and autumn. However, we found the calcium concentrations to be higher in spring and autumn. Moreover, previous studies have been unable to find any relation between calcium and season [13]. Clearly, seasonal variations in calcium concentrations have to be evaluated further.

There are several studies in the literature supporting the role of oestrogens in influencing serum calcium concentrations. Menopause [6,22], use of oestrogens, such as in oral contraceptives or for menopausal symptoms (HRT) [7], pregnancy [10] and

Table III. Crude and adjusted odds ratios (OR) for high (⩾2.35 mmol/l) *vs.* low (⩽2.34 mmol/L) calcium levels, and regression coefficients (β_i) from multiple regression analysis, in relation to reproductive history, life-style factors, and season.

Factor	Low (n)	High (n)	Crude OR (95 % CI)	Age-adjusted OR (95 % CI)	Adjusted* OR (95 % CI)	β_i (95 % CI)*
Age (years)						
⩾40– <45	522	386	1.33 (1.12–1.58)	not applicable	1.91 (1.58–2.31)	2.86 (2.12 to 3.59)
⩾45– <50	891	495	1.00	not applicable	1.00	Baseline
⩾50– <55	1036	842	1.46 (1.27–1.69)	not applicable	1.24 (1.05–1.46)	0.92 (0.27 to 3.59)
⩾55	1662	2280	2.47 (2.18–2.80)	not applicable	1.73 (1.50–2.00)	2.56 (1.99 to 3.13)
BMI (kg/m^2)						
<20	296	308	1.18 (0.99–1.40)	1.24 (1.04–1.47)	1.21 (1.01–1.45)	−0.73 (−1.43 to −0.02)
⩾20– <25	2261	1995	1.00	1.00	1.00	Baseline
⩾25– <30	1153	1201	1.18 (1.07–1.31)	1.13 (1.02–1.25)	1.10 (0.98–1.22)	−0.31 (−1.06 to 0.44)
⩾30	401	496	1.40 (1.21–1.62)	1.28 (1.11–1.49)	1.20 (1.03–1.40)	−0.25 (−1.12 to 0.61)
Age at menarche (years)						
<12	508	505	1.03 (0.90–1.17)	1.06 (0.93–1.21)	1.01 (0.88–1.16)	−0.55 (−1.10 to −0.01)
⩾12	3560	3448	1.00	1.00	1.00	Baseline
Oral contraception						
No	3814	3854	1.00	1.00	1.00	Baseline
Yes	273	115	0.42 (0.33–0.52)	0.53 (0.42–0.67)	0.67 (0.53–0.85)	−2.17 (−3.05 to −1.30)
Menopausal status						
Pre	1481	796	1.00	1.00	1.00	Baseline
Peri/post	2630	3207	2.27 (2.05–2.51)	2.02 (1.78–2.29)	2.43 (2.12–2.78)	3.88 (3.35 to 4.40)
Number of children						
0	584	685	1.27 (1.12–1.44)	1.28 (1.13–1.45)	1.35 (1.19–1.54)	1.08 (0.57 to 1.59)
1–2	2434	2244	1.00	1.00	1.00	Baseline
3–4	1013	977	1.05 (0.94–1.16)	1.06 (0.95–1.18)	1.04 (0.94–1.16)	0.15 (−0.28 to 0.59)
>4	41	42	1.11 (0.72–1.72)	1.06 (0.68–1.64)	0.91 (0.58–1.43)	−0.53 (−2.32 to 1.26)
HRT in peri/postmenopausal						
No	2014	2850	1.00	1.00	1.00	Baseline
Yes	614	356	0.41 (0.36–0.47)	0.41 (0.36–0.48)	0.42 (0.36–0.48)	−4.19 (−4.77 to −3.62)
Smoking status						
Never	1922	1895	1.00	1.00	1.00	Baseline
Ex	844	758	0.91 (0.81–1.02)	0.93 (0.82–1.04)	0.91 (0.80–1.03)	−0.31 (−0.79 to 0.17)
Current	1320	1322	1.02 (0.92–1.12)	1.07 (0.96–1.18)	1.02 (0.92–1.14)	0.00 (−0.43 to 0.42)
Alcohol consumption						
Low	2563	2440	1.00	1.00	1.00	Baseline
Medium	1166	1020	0.92 (0.83–1.02)	0.97 (0.88–1.08)	1.03 (0.92–1.14)	0.26 (−0.16 to 0.69)
High	83	92	1.16 (0.86–1.58)	1.30 (0.96–1.77)	1.35 (0.99–1.85)	0.88 (−0.37 to 2.13)
Prevalent cancer						
No	3931	3798	1.00	1.00	1.00	Baseline
Yes	180	205	1.18 (0.96–1.45)	1.10 (0.90–1.36)	1.02 (0.83–1.27)	0.29 (−0.55 to 1.14)
Screening season						
Winter	1722	1557	1.00	1.00	1.00	Baseline
Spring	778	866	1.23 (1.09–1.39)	1.20 (1.06–1.36)	1.26 (1.11–1.43)	−0.91 (−1.45 to −0.36)
Summer	1023	936	1.01 (0.90–1.13)	1.01 (0.90–1.14)	1.03 (0.91–1.16)	0.06 (−0.57 to 0.70)
Autumn	588	644	1.21 (1.06–1.38)	1.24 (1.08–1.41)	1.20 (1.04–1.38)	−0.78 (−1.40 to −0.15)

* Adjusted for all factors in the table.

the phases of the menstrual cycle [9] have been shown to affect serum calcium, with oestrogen generally lowering serum calcium concentrations. The exact mechanisms remain unclear, but the effect might be mediated by altered skeletal [23,24] and renal [25,26] sensitivity to PTH.

It is also possible that menopause *per se* induces changes in intestinal calcium absorption [27]; it has been shown that intestinal calcium absorption can be independently influenced by oestrogens even in the absence of vitamin D [28].

Hence, the interplay between serum calcium, PTH, vitamin D, oestrogen and other factors is complex, and it is possible that different mechanisms are responsible for the observed differences between calcium concentrations in this cohort. Future studies, which must include information on vitamin D and PTH status, and possibly also oestrogen, are needed in order to clarify this.

There was no association in this cohort between smoking and serum calcium, whereas at least two previous studies have found such associations [1,11]. The association between alcohol consumption and serum calcium concentrations was ambiguous in this cohort. The logistic regression analysis showed a positive association, whereas multiple regression analysis and the Bonferroni *t*-test comparing means in different groups did not reach statistical significance.

One cause of high calcium concentrations in selected populations is advanced malignancy. In our cohort, there were data on prevalent cancer for all 8,114 women, but there was no significant association between this condition and serum calcium concentrations. When subjects with prevalent cancer were excluded, results were similar.

It is questionable whether it is appropriate to use a single determination for ranking serum calcium concentrations. Both short-time [29] and long-time [30] intra-individual variation in total serum calcium are low. Even though serum calcium concentrations rise with menopause, there seems to be significant 'tracking', i.e. the ranking of calcium concentrations between individuals tends to remain the same before and after menopause [22]. Inter-individual differences in absolute values for serum calcium are low and their clinical significance might be questioned. However, in order to better understand calcium metabolism and biological mechanisms, even minor differences may be important. Since serum calcium might be a risk factor for common diseases such as cardiovascular and malignant disease, small variations in absolute concentrations might be of great importance, especially when large groups are compared. The differences in serum calcium concentrations are of the same magnitude as in previous studies [1]. Thus, we believe that a single measurement of serum calcium is a useful marker for differences with regard to calcium homeostasis.

It has been argued that free (ionized) calcium provides a better measure of calcium status, since total calcium concentrations are affected by plasma protein concentrations, notably albumin. However, total calcium has been considered a good measure of calcium homeostasis in outpatients and healthy individuals where albumin will be expected to be in the normal range [17]. In this material, among those where albumin was measured (n=2206), only 77 had values outside the reference interval of 36–45 g/L. Moreover, all samples were collected in a standardized manner, which minimizes differences in albumin concentrations due to fasting status or diurnal variation [31]. It is thus reasonable to consider total serum calcium as a useful and valid measurement of calcium status in this study population.

A valid question is whether serum calcium concentrations in this cohort can be considered representative of the general population. This cohort mainly comprised middle-aged women and 30 % of the women invited to the health examination did not attend. As there was no information about exposure to the studied risk factors in women outside this cohort, observed concentrations may not be applicable to all age groups or to the general population. However, as there was a wide distribution of calcium concentrations it was possible to make internal comparisons between subjects with low and high values, respectively. It can thus be assumed that the estimations of associations were not particularly affected by selection bias.

It is possible that both high serum calcium concentrations and reproductive and/or lifestyle factors are associated with other known or unknown factors, i.e. confounding. The multivariate analysis included all studied factors, i.e. age, reproductive factors, lifestyle, prevalent cancer at baseline and season. Hence, these factors ought not to have confounded each others' association with calcium concentrations. A limitation of the study is that there was no information on potentially important factors such as genetics, physical activity, coffee consumption, age at first birth and age at menopause, dietary intake of calcium and vitamin D or supplement use. It has previously been shown that calcium concentrations in healthy individuals are mainly unaffected by calcium intake [1]. It is difficult to predict vitamin D concentrations from dietary information, but direct measurements of vitamin D (i.e. 25-OH- vitamin D) would have been valuable. This could be the object of future studies. Polymorphisms and mutations in genes for proteins responsible for calcium homeostasis, especially the calcium-sensing receptor CASR, have been shown to be associated with serum calcium concentrations [32,33]. We are not aware of any study investigating associations between these genetic variants and reproductive factors, but this would be an interesting object for future study. The acute effects of

physical exercise include an increase in PTH and a decrease in serum calcium [34], but long-term effects are less certain [1]. In previous studies, strong but small positive associations have been found between coffee consumption and serum calcium [1]; this could not be examined in the present cohort.

This large population-based cohort study shows that reproductive factors, such as menopausal status, use of oral contraceptives or hormone-replacement therapy and age and BMI are associated with serum calcium concentrations.

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Paper III

Paper IV