CALYPSE BESSEM AGBORSANGAYA

Biomarkers for Risk of Breast Cancer during Pregnancy

A nested case control study

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UNIVERSITY OF TAMPERE

UNIVERSITY
OF TAMPERE

Dedicated to my beloved mum and dad, Bertha and Aaron Agborsangaya

"Success is not necessarily about having the best, but doing the best with what we have"-Calypso

SUMMARY

Breast cancer is a major public health problem, which is increasingly common in fertile-aged female populations, notably, also in conjunction with pregnancy (during pregnancy or one year post partum, i.e. the so called pregnancy associated breast cancer, PABC). Earlier findings indicate that breast cancer has a multi-factorial etiology, and biomarkers associated with cell differentiation and proliferation may act as potential indicators of the disease development.

We assumed that prediagnostic serum vitamin D (25-dihydroxy vitamin D, 25-OHD) levels together with endogenous (steroid) hormone expression, exposure to Epstein-Barr virus (EBV), p53 expression might be potential indictors of the PABC risk. The validity and quality of the exposure/indicator measurements were confirmed by studying the effect of storage time on vitamin D and steroid hormones in serum samples stored for many years at -25 ^{0}C.

The present study utilised molecular epidemiological tools to evaluate the role of prediagnostic biomarkers (25-OHD, EBV and p53 serological markers as well as androstenedione) on the risk of development of breast cancer, with particular emphasis on PABC, one of more aggressive sub-groups of breast cancer.

Our case-control study was nested within the Finnish Maternity Cohort (FMC), a population-based biobank of first trimester serum samples. Eligible breast cancer cases consisted of two groups; women who developed breast cancer not more than ten years (but more than 20 months) from the date of sampling, and women with breast cancer diagnosis during pregnancy or one year after date of sample withdrawal. Age and pregnancy history matched controls were sampled within the same study population. The serum samples were analysed for 25-OHD as a measure of circulating vitamin D, immunoglobulin G (IgG) antibodies specific for EBV antigens Early Antigen (EA), EBV nuclear antigen (EBNA) and EBV replication activator, the ZEBRA protein. Levels of serum IgG antibodies specific to p53 and p53 protein were also studied.

We found that serum samples stored in the FMC biobank at -25 ^{0}C for up to 24 years does not affect 25-OHD detectability, and the samples can be used to study hormone and vitamin D-disease associations. However, it is important to match cases and controls for season of sample withdrawal in vitamin D related studies.

No association was observed between vitamin D levels and risk of breast cancer in general. On the other hand, higher levels of vitamin D were associated with increased risk of breast cancer occurring during or soon after pregnancy. Previous EBV infection was not associated with increased risk of PABC, but serological EBV reactivation markers among individuals with more than sufficient levels of vitamin D were associated with a significantly increased risk of the disease.

As for the association between prediagnostic serum p53 levels and PABC risk, we found that higher levels of p53 autoantibody were associated with a modest increased risk for development of PABC, which was significant among women with higher levels of vitamin D and androstenedione.

This is the first study to provide direct epidemiological evidence on important biomarkers of the risk of PABC. The positive association between levels of vitamin D and risk of PABC remained significant among women with EBV reactivation. These observations are reminiscent of observations on other associations between EBV, vitamin D and chronic diseases, e.g. multiple schlerosis, but the underlying mechanisms remain not clear. Further studies are warranted to better understand the role and potential impact of circulating biomarkers on the risk of breast cancer occurring during or soon after pregnancy.

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Abbreviations

1, 25-$(OH)_2$D   1, 25-dihydroxyvitamin D

25-OHD   25- hydroxyvitamin D

Ab   Antibody

Ag   Antigen

ASR   Age-standardized rates

CI   confidence interval

DCIS   Ductal carcinoma in situ

EBNA   Epstein-Barr virus nuclear antigen

EBV   Epstein-Barr virus

ELISA   Enzyme-linked immunosorbent assay

FCR   Finnish Cancer Registry

FMC   Finnish Maternity Cohort

Her2-neu   human epidermal growth factor receptor 2

IDC   Invasive ductal arcinoma

Ig G   Immunoglobulin G

ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma in situ
OR	Odds Ratio
PABC	Pregnancy-associated breast cancer
RIA	Radio-immuno assay
ZEBRA	Z Epstein-Barr virus replication activator

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the list of original publications listed below:

I.   **Agborsangaya CB**, Toriola AT, Grankvist K, Surcel HM, Holl K, Parkkila S, Tuohimaa P, Lukanova A, Lehtinen M. *The effect of storage time and sampling season on the stability of vitamin D and androstenedione.* Nutrition and Cancer: An international Journal. Nutr Cancer. 2010; 62, 51- 57

II.   **Agborsangaya CB**, Surcel HM, Toriola AT, Pukkala E, Parkkila S, Tuohimaa P, Lukanova A, Lehtinen M.*Serum 25-hydroxyvitamin D at pregnancy and risk of breast cancer in a prospective study.* Eur J Cancer. 2010; 46,467- 470

III.   **Agborsangaya CB**, Lehtinen T, Toriola AT, Pukkala E, Surcel HM, Tedeschi R, Lehtinen M. *Association between Epstein-Barr virus infection and risk for development of pregnancy-associated breast cancer: Joint effect with vitamin D?* Eur J Cancer. DOI: 10.1016/j.ejca.2010.07.006.

IV.   **Agborsangaya CB,** Lehtinen T, Toriola AT, Grankvist K, Pukkala E, Surcel HM, Lehtinen M. *Serum p53 protein and p53 autoantibody expression as markers of risk of pregnancy-associated breast cancer* (submitted)

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1 Introduction

Breast cancer is the most common cancer among women both in developed and developing countries, affecting one in ten women during their lifetime (Ferlay J et al. 2010). It is also the principal cause of cancer death among women globally. For over 140 years, scientists have intensively sought to understand the epidemiology of breast cancer, principally to identify risk factors for primary prevention of the disease. Breast cancer has multi-factorial aetiology, with major contributing risk factors such as genetic predisposition, reproductive history and environmental exposures. Because of the heterogeneous origins of the disease, recent epidemiological studies assessing the risk factors of breast cancer have focused on particular disease sub-groups.

Breast cancer occurring during pregnancy or one year postpartum, also known as pregnancy-associated breast cancer (PABC) is increasingly common in affluent countries as women delay pregnancy to their 30s and 40s (Andersson et al. 2009). Although a fatal disease, ethiopathological studies on the risk markers of PABC are scarce, probably because of the relatively infrequent occurrence of the disease. The present study utilizes the unique potential of the world's largest serum biobank, the Finnish Maternity Cohort (FMC), to evaluate the association between serum biomarkers (vitamin D, endogenous hormones, Epstein-Barr virus infection, circulating p53) and the risk of development of PABC.

2   Review of the Literature

2.1   Breast cancer natural history

Breast cancer is a malignant tumour of the breast. It is a condition in which the normal cells in the breast multiply wildly because the cellular growth regulatory mechanism is defective. When localized around the tissue of its origin, breast cancer is referred to as "in situ". It is "invasive" if it spreads or metastises to distant tissues.

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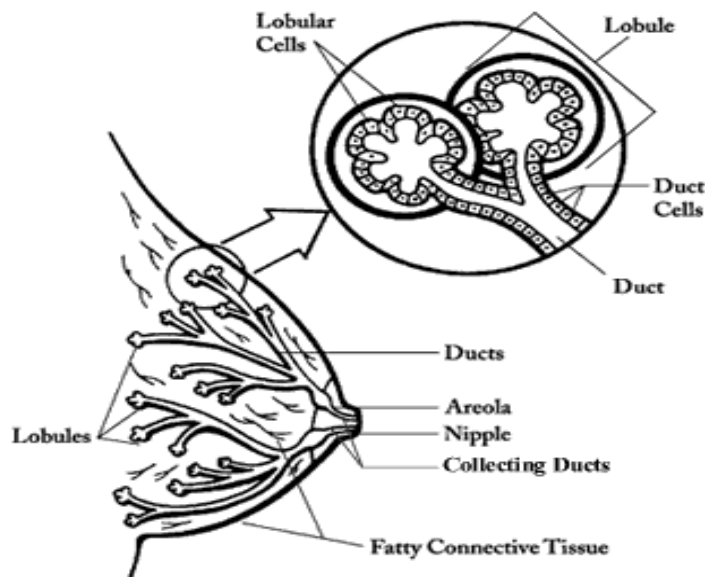
Fig 1: Illustration of the breast tissue (American Cancer Association)

The female mammary gland is a structurally dynamic organ which varies by age, menstrual cycle and reproductive status of the woman. In adulthood, it comprises of a tree-like structure of branching ducts, and the lobulo-alveolar units which arise during pregnancy (Henninghausen and Robinson 1998) (Fig 1).

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In order to understand breast cancer biology, several studies have sought to elucidate the nature of the mammary stem cell, which gives rise to the different breast cell types. A balance between cell proliferation, cell differentiation and cell death is thought to be critical for normal development (Cariati and Purushotham 2008). Perturbations in this balance probably contribute to cancer development. For instance, conditions of up-regulated cell proliferation or down-regulated apoptosis may foster accumulation of mutations, contributing to the subsequent development of breast cancer. However, it is not fully understood how normal mechanisms which control cell differentiation, cell growth and cell death in the human breast tissue act in tumour development or the protection thereof (NIH 1999, Russo 2001).

Advancements in the experimental transformation of human cells have shown that the disruption of certain regulatory pathways in the cell is sufficient to impart a tumorigenic phenotype to a wide variety of normal cells (Hahn 2002). Events leading to carcinogenesis were originally simplified (reviewed by Gescher A et al. 1998) into three distinct phases (**Fig 2**): initiation, promotion and progression. The initiation stage is thought to be rapid, involving binding and damage to DNA of a cell by a carcinogens or alterations in the stability of the normal genotype and phenotype as a result of the local collapse in the system of intercellular processes (Steen 2000). Cascades of mutations in these crucial genes may be synergistic and irreversible (McMurray et al. 2008).

At the promotion stage, there is clonal expansion of initiated cells induced by tumour promoters (e.g. mitogens) for the initiated cell. This stage is thought to be reversible and may depend on a variety of other extracellular factors, such as hormones and immunological compatibility (Steen 2000). The progression stage represents an extension of promotion whereby continuous cell proliferation caused by promoters allows the cellular damage inflicted by initiation to be further propagated.
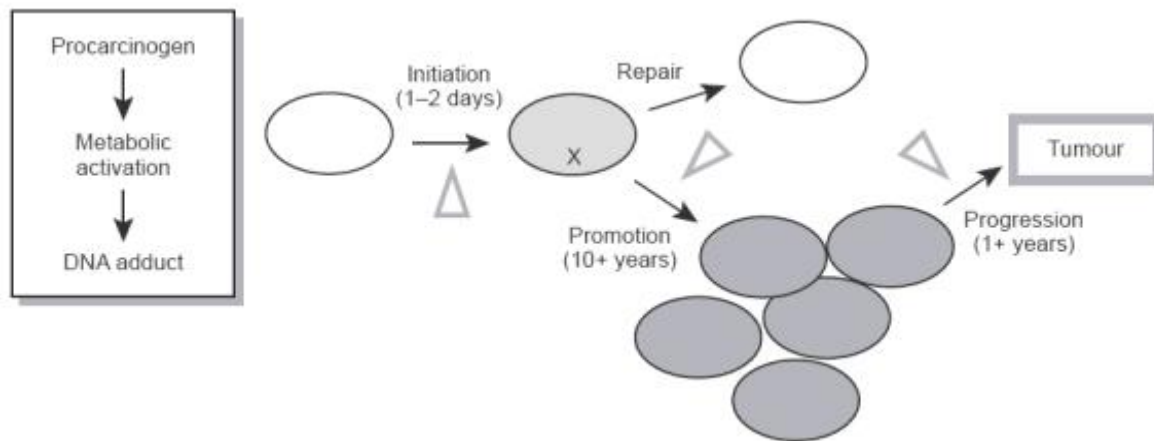
Fig 2: Hypothetical phases of multistep carcinogenesis

(Reproduced, with permission, from Gescher et al. 1998)

2.2   Breast cancer classification

Depending on the stage of the disease, breast cancer can be categorized as non-invasive or invasive breast cancer. Non-invasive breast carcinoma refers to breast cancer that is localized and has not spread (metastasized) to surrounding tissues. Major sites of origin are the ducts (tubes which drain milk from the lobules to the nipple) and lobules (site of milk production) (NBOCC 2009). There are two principal forms of non-invasive breast cancer, ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS).

Ductal carcinoma in situ, usually the most common type of non-invasive breast cancer, refers to neoplastic cells within the milk ducts of the breast, which have not spread through the walls of the ducts into the surrounding breast tissue. On the other hand, lobular carcinoma in situ (LCIS) is non-invasive breast cancer which begins in the milk-producing glands, also called the lobules (Fig 1), where it is localized. DCIS and LCIS represent the state at which there is no evidence of the spread of cancer beyond the site of origin, hence called stage 0.

Invasive (infiltrating) breast carcinoma is breast cancer that has invaded or metastasised to surrounding tissues. Depending on the stage of tumour spread, it is clinically classified as stage I (cells spread but not detected in lymph nodes), stage II,

stage III and stage IV breast cancer. Based on the site of origin, invasive breast cancer may be invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC).

IDC is the most common breast cancer subtype, constituting about 80% of all invasive breast cancers. It originates in the milk ducts of the breast and breaks through the basal membrane of the duct, then grows into the surrounding fatty tissue from where it can metastasize to other parts of the body through the circulatory (blood and lymphatic) systems. Invasive lobular carcinoma (ILC) originates in the lobules and it may also metastasize to other parts of the body. Only 10% of all invasive breast carcinoma types are ILC **(American Cancer Society)**.

A less common subtype is the inflammatory breast cancer, typically characterised by the absence of a tumour mass making it more difficult to detect by screening techniques such as mammography. It constitutes less than 3% of all breast cancers, but its incidence is on the rise (Hance et al. 2005).

Breast cancer may be categorised into other sub-types based on more detailed characteristics such as the expression of molecular biomarkers, e.g. hormone receptors (proteins on the breast cells surface that allow hormone binding) (NBOCC 2009). Examples are the oestrogen receptor, progesterone receptor, and the human epidermal growth factor receptor 2 (HER2) (Parise et al. 2009). These subgroups which show different clinicopathological and survival characteristics (Onitilo et al. 2009) have been important for patient classification in the identification of treatment options. Triple-negative breast cancer, breast cancer characterised by the absence of all three receptors, occurs more frequently in young women <50 years**,** (Haffty et al. 2006, Dent et al. 2007) and tends to have a poor response to treatment and poor prognosis (Haffty et al. 2006, Carey et al. 2007, Dent et al. 2007).

2.3   Descriptive epidemiology of breast cancer

Breast cancer in women is a primary public health concern throughout the world accounting for 16% of all female cancers. Over 1.3 million women are diagnosed with

breast cancer yearly with over 410,000 dying from the disease worldwide (Parkin and Fernández 2005, Parkin et al. 2006). In 2009 about 4.4 million women were living with the disease (The Lancet 2009). Basically, one in ten of all new cancers diagnosed each year is a cancer of the breast (Ferlay et al. 2004). The incidence rates vary markedly in the world (illustrated in Fig 3), being higher in developed countries and lower in developing countries (Stewart and Kleihues, 2003). Recent WHO data indicates age-standardized rate per 100,000 which range from 89.9 in Western Europe, 84.0 in Northern Europe, 76.6 in North America, 28.0 in Africa and 26.0 in Asia. Still within continents, there is marked variation. In Africa for instance, the incidence is 27.1 and 8.0 per 100,000 in Cameroon and the Gambia, respectively (GLOBOCAN 2008).

Recent trends in developing countries indicate an increase in incidence of breast cancer especially in urban regions (WHO Global Burden of Disease 2008). It is the leading cause of cancer in Southeast Asian women, second only to gastric cancer in East Asian women and cervical cancer in women in South-Central Asia (Wong 2009). For instance in India, 100 000 women are diagnosed with breast cancer annually, and a rise to 131 000 cases per year is predicted by 2020. Similar trends have been observed in other developing countries, which are projected to constitute most of the 26% increase in breast cancer incidence predicted by 2020 (Stewart and Kleihues, 2003). These changing patterns in breast cancer incidence are generally attributed to "westernization", a term for lifestyle changes associated with increased breast cancer risk such as nulliparity, postponed childbearing and breastfeeding, increased exogenous hormonal use, as well as increased physical inactivity alongside obesity.

Estimated age-standardised incidence rate per 100,000

Breast, all ages

■ < 21.3   ■ < 28.3   ■ < 38.9   ■ < 56.8   ■ < 109.4

GLOBOCAN 2008 (IARC) - 10.6.2010
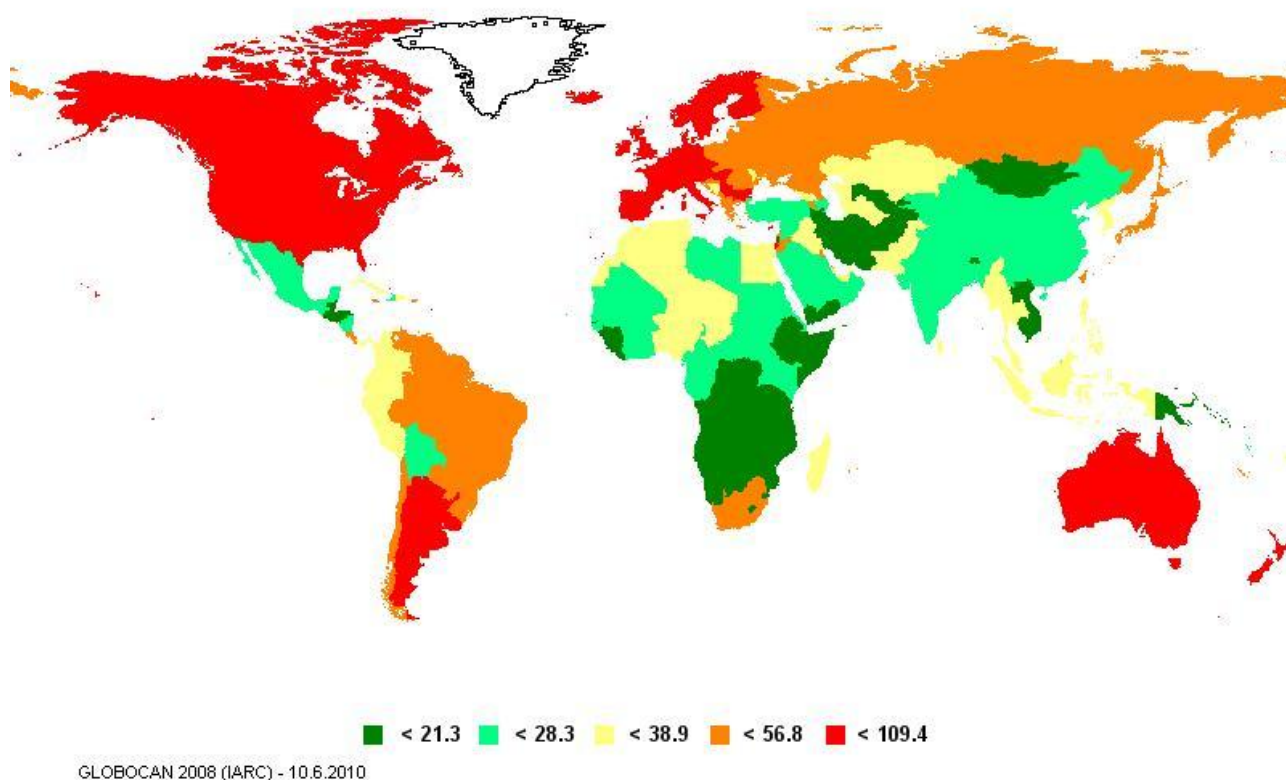
Fig 3: World-wide incidence of breast cancer for 2008

Breast cancer mortality has been on a decline worldwide over the last two decades, probably due to the introduction of population-based mammographic screening of women aged 50-69, which is associated with early detection of the disease (Smith 2002). This decline is especially evident in developed countries, where screening was introduced since the mid-80. Screening is thought to increase opportunity for detection of early stage cancers, which results in favourable prognosis. In concert with improved management, this has led to reduced mortality from the disease (Liu et al. 2010). Moreover, effective adjuvant therapies employing systemic and hormonal agents reduce recurrence of local or nodal disease (Berry et al. 2005). In the Nordic countries, the female breast cancer mortality reduced from over 19 to 16 deaths per 100,000 per year from the 1980 to 2005 (NORDCAN).

In Finland, the incidence of breast cancer has been steadily increasing long before screening was introduced in 1987 (Botha et al. 2003), particularly when compared to other cancers (Fig 4). Recent data from the Finnish Cancer Registry (FCR), the national cancer registry in Finland, shows ASR of 89.2 per 100 000 with a total of 4323 new cases in 2008, compared to 45.4 per 100,000 with 1583 new cases in the late 1970s.

Despite the increasing incidence of breast cancer, Finland has experienced a marked increase in the survival rate, with a 5-year survival rate of 89% in 2007 (FCR, 2010) compared to less than 76% in the 1970s (Karjalainen 1990). The better survival may be attributed to improved early diagnosis following the introduction of the mammographic screening for women aged 50-69 years and advancement in treatment methods.

Mammography for breast cancer screening has been shown to be efficient in screening women aged 50-69 years, with little evidence on its efficacy in ages 30-49 years (Vainio et al. 2002). It is effective in the detection of in situ and early stage cancers which are both indolent but not in the detection of aggressive cancers (Esserman et al. 2007). There is a need for biomarkers to better discern the risk of biologically aggressive breast cancers, particularly when it coincides with a physiological state such as pregnancy that may mask the disease during screening.

Finland
Incidence: ASR (World), Female age (0-85+)
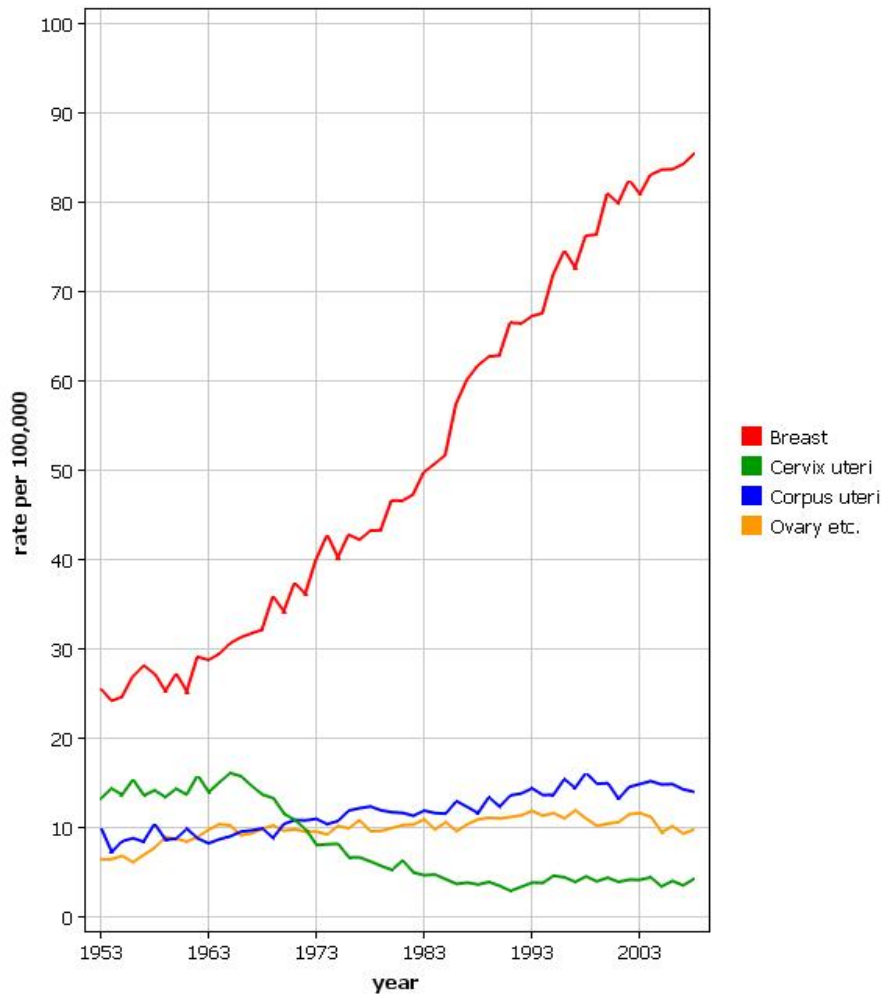
Breast
Cervix uteri
Corpus uteri
Ovary etc.

Fig 4: Age-adjusted incidence trends of selected female cancers in Finland from 1954 – 2003

Due to its heterogeneous nature of the disease, recent studies on breast cancer have been confined to specific subgroups. The present study, although entails a non-pregnant subgroup of breast cancer, predominantly evaluates risk markers of pregnancy-associated breast cancer (PABC).

3 Pregnancy-associated breast cancer (PABC)

PABC has been variably defined as breast cancer occurring during gestation, lactation or within one year from delivery. Lactation is defined as 12 months following parturition, regardless of whether the mother nurses her baby or not (Barthelmes et al. 2005).

3.1 PABC natural history

Pathologically, 75 – 90% PABC is of the invasive ductal carcinoma sub-type, followed by invasive lobular carcinoma. Inflammatory sub-types are generally rare among PABC ranging from 1.5 – 4% (Bonnier et al. 1997). Around 70% of all PABC tumors are estrogen and progesterone receptor negative, whereas 30 – 60% of the tumors express HER2/neu (Ishida et al. 1992, Merkel 1996). PABC cases generally present an advanced stage and larger tumor size at diagnosis (Kroman and Mouridsen 2003, Rodrigues et al. 2008). This is consistent with a poor prognosis, when compared to other breast cancer types (Anderson et al. 1996, Rodrigues et al. 2008).

The advanced stage at diagnosis may be attributed to delayed diagnosis associated with physiologic changes in the breast tissue. Pregnancy and breastfeeding increase the breast density because of engorged blood vessels, making mammography and clinical examinations difficult to interpret. An alternative hypothesis is that the gestational hormones during pregnancy account for the poor prognosis of pregnancy-associated breast cancer. These hormones are thought to increase the risk of occurrence and worsen the prognosis due to their growth-promoting effects (Pathak 2002). The high endogenous steroid and other hormone milieu presented during pregnancy due to increased levels of estrogen, progesterone and insulin-like growth factor may foster a more aggressive tumor growth (Schedin 2006).

3.2   Occurrence of PABC

Breast cancer is the most common cancer occurring during pregnancy, with incidence rates ranging from 1 in 10,000 to 1 in 3,000 pregnancies (Lambe and Ekbom 1995). This constitutes up to 6 % of all breast cancers diagnosed in fertile women under the age of 45 in USA (Johannsson et al. 1998, Psyrri and Burtness 2005). Having the first pregnancy at the age of 30 years or older, and an increased maternal age are established independent risk factors for breast cancer (Psyrri and Burtness 2005). As women elect to delay child bearing to later decades of their lives, the incidence of breast cancer is expected to rise among obstetric populations. A recent study in Sweden showed that the incidence of PABC doubled, from 16 to 37.4 per 100,000 pregnancies, from 1963-1974 to 1990-2002 (Andersson et al. 2009).

Some studies reported no excess risk for breast cancer at pregnancy by comparing the observed number of PABC to the expected number at population level using age-standardized incidence rates (Harris et al 1992, Andersson et al. 2009). The results were, however, hampered by the calculations based on linkage of birth and cancer registries. Thus, women who gave birth to a live-born only were included, and women diagnosed at the first and second trimesters, who most likely underwent induced or spontaneous abortion were excluded. In Europe, the average gestational age at PABC diagnosis is 21 weeks (Aebi and Loibl 2008). The diagnosis is made in 21.6% of cases during the first trimester, 43.3% of cases during the second trimester, and 35.1% of cases during the third trimester (Aebi and Loibl 2008). The use of a population-based cohort that samples women routinely irrespective of the outcome of pregnancy is important. Given that the incidence of PABC is increasing, there is a need to better understand the etiology of this disease.

4   Risk factors of breast cancer with specific reference to PABC

A risk factor is anything, ranging from a lifestyle choice to an inherent characteristic that influences a person's chances of developing a disease (NBOCC 2009). While the definite cause of breast cancer is far from being unraveled, some factors have been associated with increased risk of developing the disease.

Overall, Breast cancer is predominantly a female disease, with only 1 % of the cases occurring in men (Fentiman et al. 2006). Thus, women have an increased risk of developing the disease during their lifetime compared to men. Other factors that are associated with risk of breast cancer and PABC can generally be distinguished into non-modifiable and modifiable risk factors.

4.1   Non-modifiable risk factors

Non-modifiable risk factors constitute characteristics that cannot be changed, and are usually present independent of the individual's actions.

4.1.1 *Age*

Apart from the female gender, increasing age is the single strongest risk factor of breast cancer in the population. It is a rare disease before age of 30, after which the incidence rises steeply with increasing age up to about the age of 50 years (Greenle et al. 2000). Thereafter, incidence still increases with age, but at a slower rate. The strong dependence of breast cancer incidence on age, apart from the accumulating genetic damage that occurs during the lifespan, has long been shown to correlate with the duration of ovarian hormone exposure (Pike et al. 1983). Animal studies show that elevated hormonal stimulation increases occurrence of mammary carcinogenesis in rodents (Russo and Russo 1996).

Predictions indicating an increase in the incidence of the disease are based on the fact that PABC is age-related. The reported mean age at diagnosis ranges from 32 to 34 years (Middleton et al. 2003, Hahn et al. 2006, Cardonick et al. 2010). Women who have their first full term pregnancy after the age of 30 show up to 3-fold increased risk for development of breast cancer compared to those at ages less than 20 (Middleton et al. 2003). Studies are warranted to investigate the effect of age, for instance as an independent risk factor, on the risk of development of PABC.

4.1.2 *Familial and genetic risk factors*

Family history of breast cancer is an established risk factor of the disease. About 18% of breast cancers occur in women who have a history of the disease in a first degree relative such as daughters, mother or sisters (Slattery and Kerber 1993, Collaborative Group on Hormonal Factors in Breast Cancer 2001).

It is not clear whether a family history of breast cancer is more common among PABC individuals compared to other breast cancer subgroups. An earlier Saudi-Arabian hospital based-study (Ibrahim et al. 2000) reported a significantly higher positive family history among PABC individuals than non-pregnant breast cancer patients. The definition of PABC in that study excluded those diagnosed within one year of pregnancy. A more recent study found no significant difference in family history between PABC and non-PABC patients (Beadle et al. 2009). Because previous studies depend on patient reports, population-based studies using cancer registries with a possibility to link patients within family pedigree are warranted.

Genetic studies have shown that a greater proportion of the familial breast cancer is due to specific germ line mutations within a family pedigree. Depending on the probability of disease occurrence, the genes associated with increased breast cancer may be low penetrance or high penetrance genes. An example of high penetrance genes are the BRCA 1 and BRCA 2 genes whose mutation accounts for about 20% of the familial aggregation of breast cancer, and have been associated with inheritable susceptibility to breast and ovarian cancer (Brody and Biesecker 1998). Heritable

breast cancers account for 5 – 10 % of all breast cancers, half of which are related to BRCA 1/2 gene mutations (Newman et al. 1998, Hemminki et al. 2002). In Finland, up to 21% of the breast cancer families were accounted for by mutations in these two genes (Vehmanen et al. 1997).

Mutations in the BRCA 1/ 2 genes occurs quite infrequently among PABC cases. A small population-based Swedish study showed that PABC is relatively uncommon among women harbouring mutation in the BRCA genes (Johannsson et al. 1998). In one study (Siegelmann-Danieli et al. 2003), BRCA 1/2 gene mutation carriers were less common among PABC cases than in the non-pregnant control group.

4.1.3 *Mammographic breast density*

The extent of radiological dense breast tissue (mammographic density) usually reflects differences in breast tissue composition which varies among women. Mammographic breast density is a predictor of breast cancer risk and may be an early marker of breast cancer (Boyd et al. 1998, Stone et al. 2010). One study found that women with more than 75% breast density showed over 9-fold increased risk of developing atypical hyperplasia (abnormal cell increase) or breast cancer in situ (BCIS) compared to women with no breast density (Boyd et al. 1992). In another study, 17 of 22 ductal carcinoma in situ (DCIS) tumors arose in the mammographic quadrant with the highest density (Ursin et al. 2005). Because mammography is made routinely only among women aged 50 years or older, practically nothing is known about PABC associated screening mammography changes.

4.2   Reproductive and menstrual risk factors

Surrogate measures of endogenous hormone exposure such as early menarche, late menopause, low parity and late age at first birth have been linked with increased risk of developing breast cancer in premenopausal women.

4.2.1 *Early menarche and late menopause*

Women who are exposed to endogenous sex hormones over a longer period of their life time turn to have increased risk of developing breast cancer. The concept of hormonal carcinogenesis is consistent with epidemiological observations that late menarche and early menopause have a protective effect against breast cancer (Shin et al 2010). This is consistent with the fact that late menarche (onset of menstruation) and/or early menopause (before age of 55 years) have fewer numbers of menstrual cycles and therefore shorter exposure to ovarian hormones during the reproductive years compared to women who have early menarche and/or late menopause. This also explains the observed protective effect of oophorectomy on subsequent risk of breast cancer (Chang-Claude et al. 2007). In their hospital-based patient review, Ibrahim and colleagues (Ibrahim et al. 2000) found no difference in the age at menarche for PABC and controls.

4.2.2 *Child birth*

Young age at first full-term pregnancy (FFTP) and high parity are both associated with decreased breast cancer risk over a long-term. Compared to nulliparous women, women with a full time pregnancy experience an age-dependent lifetime reduction in breast cancer risk (Ma et al. 2010). Prior to pregnancy, the cells of the mammary gland are in a vulnerable undifferentiated state, and differentiate to functioning milk-producing structures during pregnancy. Pregnancy, and subsequently FFTP may induce a specific pattern in human breast, decreasing the pool of vulnerable breast cells, as they attain full differentiation (Russo et al. 2008). The risk of breast cancer turns to increase in the first five years after delivery, probably due to the promoting effect of the hormonal milieu at pregnancy (Liu et al. 2002, Pathak 2002).

The protective effect of pregnancy on the risk of subsequent development of breast cancer over a long duration has been established. There is evidence that this protection is preceded by a transient increase in breast cancer risk following pregnancy, thus the dual effect of pregnancy on breast cancer risk (Lambe et al. 1994, Liu et al. 2002). This

is consistent with results from experimental studies which show that pregnancy induces transient alteration, followed by permanent structural changes in animal breast tissue and gene expression (transcription profile) (Russo et al. 1990a, Russo et al. 1990b). The short term increase in the breast cancer risk probably reflects a growth-enhancing effect of high endogenous hormone levels during pregnancy on tumor cells whose malignant transformation has already been initiated (Henderson and Bernstein 1991). Based on the evidence from laboratory studies, some hypotheses have been put forward to explain the long-term protective effect of pregnancy on the risk of breast cancer.

Russo and Russo proposed that the protective effect is due to terminal differentiation of the mammary epithelia driven by the pregnancy hormone, human chorionic gonadotropin (hCG) (Russo et al. 1990a, Russo et al. 1990b). This limits the population of susceptible cells which are potential targets for carcinogenesis.

Sivaraman and colleagues (Sivaraman et al. 2001) postulated the cell-fate hypothesis that the hormonal milleur during pregnancy sets on a molecular switch during pregnancy that gives rise to persistent changes in gene expression and/or signal transduction (Sivaraman et al. 2001).

Wagner and colleagues (Wagner et al. 2002) also proposed that during pregnancy, a new population of mammary epithelial cells arise from differentiating cells which fail to undergo apoptosis (cell death) during involution and may function as alveolar progenitors in subsequent pregnancies (Wagner et al. 2002). Later findings have however, contested the fact that proportion of mammalial epithelial progenitor/ stem cells change following pregnancy (Britt et al. 2009), or that observed changes are independent of pregnancy (Booth et al. 2007).

Recent prospective epidemiological studies by Toniolo and colleagues have shown that the effect of hCG, though protective for postmenopausal breast cancer (Lukanova et al. 2008, Toniolo 2010), may actually be associated with increased risk of development of the disease soon after pregnancy (Toniolo et al. 2010). On this basis, it is logical that the association between prediagnostic serum hCG levels and PABC is positive, although it is remains to be tested.

4.2.3 *Previous exposure to radiation*

Frequent exposure to ionizing radiation from diagnostic X-rays is an established risk factor of breast cancer (Boice et al. 1991, Ronckers et al. 2008). If exposed at a younger age, the lifetime risk of breast cancer is much higher. This risk is especially thought to be highest at puberty and the period surrounding the first pregnancy, when breast ductal cells are actively developing (Ronckers et al. 2005). This increased risk usually remains throughout the lifetime (Preston et al. 2002).

There is substantial evidence for increased breast cancer risk from studies of female Hodgkin lymphoma survivors (Travis et al 2005, Alm El-Din et al 2008). In female populations with a familial or genetic predisposition to breast cancer, there is evidence that yearly exposure to low dose ionizing radiation from mammography increases breast cancer risk (Jansen-van der Weide et al. 2010).

With regards to PABC, there is no indication that previous exposure to radiation is associated with a disease occurrence of the disease. However, since women exposed to radiation at a younger age are at increased risk of developing breast cancer, it is plausible that the diagnosis of breast cancer within this population coincides with subsequent pregnancies.

4.3 Lifestyle and Environmental risk factors

There has been increased interest by researchers to understand the risk factors of breast cancer that are associated with the environment, with the aim of preventing the disease through behavioural modification. Unlike the case of cigarette smoking and lung cancer, no single factor with a major effect on the risk of breast cancer has, however, been identified, but lifestyle and environmental factors have been moderately associated with risk of developing the disease.

4.3.1 *Diet*

In case–control studies of food consumption and breast cancer incidence, a clear association between patterns of food consumption and breast cancer risk has been observed (Wakai et al. 2000, Brennan et al. 2010). The effect of diet on the risk of breast cancer is probably exerted via its influence on hormone metabolism. Epidemiological evidence indicates that the nutrition determines the levels of circulating estrogen, and may affect the risk of breast cancer (Wu et al. 1999).

The ideal condition to determine the risk of breast cancer associated with a dietary exposure is to compare an intervention group with a control group which is restricted from related exposures (null contamination). In the Women's Health Initiative Dietary Modification trial, a randomized, controlled, primary prevention trial with over forty-eight thousand participants, Rohan and colleagues (Rohan et al. 2009) showed that a modest reduction in fat intake plus an increase in fruit, vegetable, and grain intake did not significantly alter the risk of benign proliferative breast disease.

4.3.2 *Alcohol*

Several studies have linked intake of alcohol and risk of breast cancer (Terry et al. 2006, Beasley et al. 2010). Meta-analyses indicate a linear increase in the risk of breast cancer with about 10% increase for each 10 grams/day increment of ethanol intake (Smith-Warner et al. 1998, Collaborative Group on Hormonal Factors in Breast

Cancer 2002, Key et al. 2006). The alcohol-associated increased risk for postmenopausal breast cancer is probably due to the accumulation of adipose tissue, which later serves as a site of production of endogenous hormones associated with increased risk of breast cancer (Maskarinec et al. 2006). On the other hand, the positive association between alcohol intake and breast cancer risk in premenopausal women (Friedenreich et al. 1993, Rohan et al. 2000), may be due to elevated circulating androgens associated with increased alcohol intake (Dorgan et al. 1994). Elevated androgen levels are associated with increased risk of premenopausal breast cancer (Kaaks et al. 2005). Although circulating levels of estrogens during pregnancy are positively associated with intake of alcohol (Petridou et al. 1992), the effect of alcohol intake before or during pregnancy and risk of PABC has not been investigated.

4.3.3 *Exogenous hormones*

Although hormone replacement therapy was recommended for menopausal symptoms decades ago, it was nearly 2 decades later that reports started to appear linking HRT with increased incidence of breast cancer (Colditz et al. 1990, Steinberg et al. 1991). In the US, nearly 40% of postmenopausal women used hormone replacement therapy (HRT) for the prevention of osteoporosis and the control of menopausal symptoms by 1995 (Keating et al.1999). Notwithstanding, it is a long established fact that HRT increases the risk of postmenopausal breast cancer (Ross et al. 2000), and the risk is thought to vary with the formulation and preparation of HRT (Opatrny et al. 2008).

On the other hand, the use of oral contraceptives (OC) is very common with more than 100 million women worldwide using OCs according to a 2005 *Morbidity and Mortality Weekly Report* (Bensyl et al. 2005). Despite the fact that the estrogen-progesterone content of OCs has been greatly reduced ever since it was introduced over 40 years ago, the International Agency for Research on Cancer (IARC) in 2005 classified the estrogen-progestogen OCs as a group 1 carcinogen, indicating that there is sufficient evidence implicating them as carcinogenic to humans (Schneider et al. 2005). The reduction in the estrogen-progesterone content of OC in recent years warrants further research which takes into consideration the proportion of sex hormone content,

together with the specific duration of exposure. For PABC (with late onset of pregnancy) the prolonged use of OC may increase the risk of development of the disease, particularly for populations exposed to high estrogen-progesterone OC. This remains to be studied.

4.4   Other lifestyle factors

Findings from migrant studies suggest that increase in breast-cancer incidence following migration from countries with low breast cancer incidence to higher-incidence countries support the role of changes in lifestyle on breast cancer risk alteration. It has, however, been difficult to disentangle the individual specific factors (Ziegler et al. 1993). Data supporting the role of factors such as high fat intake, low soy intake and low vegetable intake has been largely inconclusive. In a nutritional questionnaire-based case control study of women in Shanghai China, an aggregate "meat–sweet" diet, a surrogate of a westernized diet, was associated with a modest increased risk of breast cancer compared with a more traditional vegetable-rich diet (Cui et al. 2007). The risk of breast cancer is higher in postmenopausal obese women, possibly because of the peripheral production of estrogens in adipose tissues (Smith 2002). The risk turns to decrease with increased physical exercise (IARC 2007).

Among premenopausal women, no positive association was found for BMI with breast cancer risk (IARC 2002) probably because most oestrogen, produced by the ovaries, is homeostatically regulated by a negative feedback system involving follicle-stimulating hormone and luteinising hormone. Oestrogen levels in premenopausal women are therefore not directly determined by the levels of adipose tissue. On the other hand, obesity in premenopausal women may be associated with a slight decrease in risk for breast cancer (IARC 2002), probably due to the infrequent menstrual cycles, thus, reduced exposure to ovarian hormones. One study showed that pregnancy weight gain is associated with a reduction in risk of premenopausal breast cancer (Hilakivi-Clarke 2005).

Apart from the challenge of disentangling the accrued life-time potentially modifiable environmental and lifestyle factors associated with increased risk of breast, there is need for continued research to further refine how timing and degree of these exposures in early childhood and adolescence relate to adult breast cancer risk. For instance, early age at menarche, an important reproductive risk factor for breast cancer, is associated with higher energy intake exercise and body mass profile (Koprowski et al. 1999). A decrease in age at menarche may increase breast cancer rates in lower-resource countries where diet and exercise become patterned to the more docile western life style (Meyer et al. 1990, Koprowski et al. 1999). Further studies are therefore needed to better understand the role of these early life habits on the risk of breast cancer development in older age.

5 Biomarkers for risk of pregnancy-associated breast cancer

Age at first birth, parity and age at menarche are surrogate measures of the exposure of circulating endogenous biomarkers in breast cancer epidemiology studies. Advancement in molecular epidemiology provides the opportunity to assess more precise risk indicators such as hormone levels for reproductive status, nutritional markers for self-reported intake, viral proteins and their related antibodies indicating viral infection status. This is particularly important in understanding the risk markers of PABC, which is particularly challenging to clinicians and patients alike. The time frame between the initiation or progression of the disease and the cancer diagnosis provides a time window where biomarkers of risk may be relevant (Langseth et al. 2010).

5.1 Steroid Hormones

The positive association between steroid hormones such as estrogens and progesterone and breast carcinogenesis has long been established (Key 1995, Bernstein 2002) and forms the rationale underlying the benefits of endogenous therapies in the treatment of breast cancer (Early Breast Cancer Trialists' Collaborative Group 2005). For instance, the use of tamoxifen, the non-steroidal agent with anti-estrogenic properties, and aromatase-inhibitor therapies have markedly reduced the rate of breast cancer recurrence in patients with hormone-receptor positive tumours (Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists' Group 2008) and reduced its incidence in premenopausal female populations (Visvanathan et al. 2009).

Attempts to use tamoxifen for the primary chemoprevention of breast cancer in populations at high risk of the disease produced promising results within the *National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 trial* (Fisher 1998). Two smaller trials, the *Italian Tamoxifen Prevention Study* (Veronesi et al. 1998) and the *Royal Marsden Hospital Tamoxifen Randomized Chemoprevention Trial* (Powles et al. 1998) did not show evidence of reduction in the incidence of breast cancer following up

to five years of oral tamoxifen administration. Later reports from these trials indicated adverse effects such as increased risk for endometrial cancer, deep vein thrombosis, pulmonary emboli and cataracts (Levine et al. 2001). With the use of the Gail model (Gail et al. 1999), these interventions are limited to female populations at higher risk of breast cancer. It is possible that the effect of this chemopreventive intervention is more effective among female populations with increased risk of PABC. This remains to be studied.

Prospective studies evaluating the association between endogenous hormone levels and risk of breast cancer in premenopausal women have produced inconsistent results (Thomas et al. 1997, Kaaks et al. 2005, Eliassen et al. 2006), probably due to cyclic variations of the sex hormone levels during the menstrual cycle.

A more compelling hypothesis is to evaluate the role of circulating sex steroids at pregnancy, primarily because endogenous hormone levels increase during this window of life (Johansson 1979), and turn to promote differentiation and proliferation in the ductal and lobular-alveolar epithelium of the breast cells (Russo et al. 1982*).* Again, the higher levels of estrogens and progesterone may trigger increased proliferation of already malignant cells in the short term, thus increasing the risk of breast cancer during or shortly after pregnancy (Miller 1993).

Earlier experimental studies by Russo and Russo implicating hCG in the protective effect of pregnancy hormones on the risk of breast cancer (Russo et al. 1990a, Russo et al. 1990b) has not been corroborated by epidemiological evidence. Toniolo and colleagues showed that the effect of hCG, though protective for postmenopausal breast cancer (Lukanova et al. 2008, Toniolo et al. 2010), may actually be associated with increased risk of development of the disease just after pregnancy (Toniolo et al. 2010). On this basis, it is logical that the association between prediagnostic serum hCG levels and PABC is positive. However, no study has prospectively reported the association between circulating endogenous hormones at pregnancy and risk of PABC, probably because of the relative rarity of this breast cancer sub-entity.

Increased understanding of the association between circulating hormone levels and subsequent risk of breast cancer occurring shortly after pregnancy may provide insight into the aetiology of this disease.

Androstenedione, is a precursor of testosterone and estrogens, produced either from dehydroepiandrostenedione or 17-hydroxyprogesterone. It is therefore natural that the levels of androstenedione would reflect the levels of these hormones in circulation.

5.2   Vitamins

The association between vitamin intake and risk of breast cancer, though decades-old, is inconclusive. While some reports indicate an increase in the risk of breast cancer by up to 30% associated with multivitamin intake (Berube et al. 2008, Larsson et al. 2010), others show a breast cancer risk reduction between 20-40% (Gaudet et al. 2004, Webb et al. 2004), whereas some show no association (Zhang et al. 1999, Dorjgochoo et al. 2008). The disparity in the results may be explained partly by the use of questionnaires with possibilities of introduction of recall bias. Moreover, multivitamin intake is only a surrogate of the circulating levels of these vitamins, and may be confounded by other physiological factors affecting absorption, transportation and the biological activity of the vitamins. This explains the growing interest in the more representative prospective studies based on prediagnostic endogenous levels of specific vitamins such as vitamin D.

Vitamin D

Vitamin D is a fat-soluble sercosteriod, primarily known for its role in maintaining calcium homeostasis as well as bone development and maintenance. Unlike the name suggests, vitamin D is not a typical vitamin because individuals with adequate sunlight exposure do not require dietary supplementation, owing to its endogenously synthesis.

Vitamin D can be obtained in two distinct ways, either through dietary intake (foods such as fish oil, mushroom, fatty fish and vitamin D fortified foods as milk) or through

endogenous synthesis. Exposure of the skin to solar UVB (range, 280-315 nm) or artificial sources, photolyses 7-dehydrocholesterol to pre-vitamin D, further hydroxylated to 25-hydroxyvitamin D {25(OH)D } in the liver, and then to 1,25-dihydroxyvitamin D {1,25-OH_2D } within the kidney and other tissues (Holick 2003).

Endogenous synthesis of vitamin D constitutes between 80- 90 % of the total vitamin D sources in western European populations (Willet 2005, Holick 2004). This is usually different across population groups and between individuals because of wide differences in skin exposure to UVB radiation, the efficiency of cutaneous synthesis, and food fortification practices (Prentice 2008). Other factors such as clothing habit and sunscreen use affect UVB skin exposure (Webb 2006).

The hormonal form,1,25-OH_2D acts in a wide range of tissues by binding to the nuclear vitamin D receptor (VDR), present in cells of organs such as liver, pancreas, brain, lung, skin, muscle, adipose tissue, and breast (Al-oanzi et al. 2006). The binding of this hormone unto VDR forms a complex that regulates gene transcription. This is known as the genomic pathway of vitamin D action. The non-genomic action of vitamin D entails the more rapid non-transcriptional responses in which there is activation of trans-membrane signal transduction pathways (De Boland and Boland 1994).

The less active form, 25(OH)D, is a useful marker of the overall vitamin D status because it is relatively stable with a half-life of about 2-3 weeks, and its concentration is not under tight homeostatic regulation, as is the more active form 1,25$(OH)_2$D (Lund et al.1980).

At vitamin D deficiency, sufficient levels can be obtained by intake of vitamin D fortified foods or supplements. On the other hand, hypervitaminosis D, characterized by circulation 1,25$(OH)_2$D is excess, triggers a degradation pathway via the enzyme 24,25-hydroxylase. This metabolises the 1, 25-$(OH)_2$D to 24, 25-dihydroxyvitamin D, which is further catabolised to calcitroic acid.

Ecological studies have shown that breast cancer incidence and mortality tend to be higher in areas with low winter sunlight levels and lower in sunny areas (Garland et al. 1990, Gorham et al. 1990, Grant 2003). Women regularly exposed to sunlight, and

consumers of above-average amounts of vitamin D, show significantly lower incidence rates of breast cancer (John et al. 1999). These trends are corroborated by strong evidence from laboratory studies that vitamin D is anti-apoptotic, supports differentiation and reduces proliferation of normal and breast cancer cells (Colston and Hansen 2002, Welsh et al. 2003, Lowe et al. 2003).

Despite the strong ecological and experimental evidence associating vitamin D and breast cancer, the International Agency for Research on Cancer concluded that the supporting evidence of the association is weak (IARC 2008) because of the inconsistent results from prospective epidemiological studies, even after the IARC publication (Freedman et al. 2008, McCullough et al. 2009).

By menopausal status, the findings have been inconsistent among postmenopausal women (Freedman et al. 2008, McCullough et al. 2009, Almquist et al. 2010) and lack of an association in premenopausal women (Hiatt et al. 1998, Almquist et al. 2010). In the Women's Health Initiative Dietary Modification trial, the authors showed that calcium plus vitamin D supplementation was not associated with the risk of benign proliferative breast disease (Rohan et al. 2008), a condition associated with increased risk of breast cancer in women. Considering the possibility of a trial of a vitamin D supplement to prevent breast cancer, the issues of timing, when to initiate supplementation, and how long it should be given, looms large (Fairfield and Stampfer 2007).

In pregnant and lactating women, a high prevalence of vitamin D insufficiency has been documented, particularly in high-risk groups (Bodnar et al. 2007, Dawodu and Wagner 2007). Vitamin D has anti-proliferative and pro-apoptotic properties, coupled with the rapidly differentiating and maturing cells during pregnancy, there is need to understand the effects of vitamin D status on the risk of development of PABC. No study has prospectively evaluated the role of vitamin D on the risk of breast cancer during or soon after pregnancy. Also, previous studies evaluating the role of vitamin D on breast cancer risk are limited to single point time measurements, which may not reflect the vitamin D acquisition over time.

5.3   Viruses

Ever since the hypothesis that mammary tumour agent in mice, later described as mouse mammary tumour virus (MMTV), may be implicated in breast cancer aetiology by John Bittner in the 1930s (Bittner 1957) , epidemiological evidence implicating viral oncogenesis in breast cancer risk has been debated. In recent years, interest has been focused on the Human papilloma virus (HPV), MMTV and Epstein-Barr virus (EBV).

EBV is a ubiquitous human gamma-herpes virus which infects more than 90% of the population worldwide. It was classified as a class 1 carcinogen by the International Agency for Research on Cancer (IARC) (Griffin 2000) because of its role in other cancer forms such as African Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal and gastric carcinomas. The association between EBV and breast cancer has been controversial because while some studies report no detection of the EBV genome in breast cancer tissues (Hemminki and Czene 1999, Chu et al. 2001, Murray et al. 2003), others report detection rates ranging from 20 to 60 % (Labrecque et al. 1995, Bonnet M et al. 1999, Fawzy et al. 2008).

Disparity in the results might be due to either differences in the methods of detecting EBV in different studies, or to the argument that EBV is confined to lymphocytes located in breast tumours (Herrmann and Niedobitek 2003). Micro-dissection and isolation of pure tumor cells have revealed that even in EBV-positive tumour samples, many tumour cells do not contain the EBV genomes and that breast carcinomas are highly heterogeneous in terms of genome content and distribution (Perrigoue et al. 2005). These findings raise the possibility that although EBV is not likely to be an etiological agent in breast carcinogenesis, the virus might contribute to tumour progression (Arbach et al. 2006).

The mechanism that favours foetal implantation at pregnancy is thought to be associated with an impaired cellular immunity in which the resistance to growing foetus is disfavoured, accompanied by a depressed circulating T lymphocyte (Nakamura et al. 1993). The impaired cellular immunity probably favours EBV infection and/or reactivation during pregnancy. Following EBV reactivation, there is expression of the latent infection membrane protein 1 (LMP1) which has oncogene-like effects. The

LMP1 signalling mediated alterations via the tumour necrosis factor superfamily alters gene expression which is critical for long term cell proliferation and survival, significant features for the development of malignancies (Izumi 2010).

5.4　Oncogens and Oncoproteins

Breast cancer is thought to be a series of stepwise genetic alterations of normal host cells or possibly from other non-genetic changes in the behaviour of host cells. These alterations result in the formation of oncogenes, a class of genes whose activation is important in the development of the cancer. The activation could result in synthesis of protein that results in increased cell growth, as in the Human Epidemal growth factor 2, *HER's-2,* which is expressed in over 20% of primary breast cancer cases (Osborne C, 2004).

On the other hand, tumor suppressor genes are very important in the regulation of cell growth. These genes are usually negative regulators of growth or other functions that may affect the invasive and metastatic potential of malignant cells, such as cell adhesion and regulation of protease activity. The loss of function within these genes results in the promotion of malignancy. Sometimes rather than a mutation of the tumor suppressor gene, there may be another mechanism that interferes with its expression or function, resulting in the synthesis of a defective protein. An example of a tumour suppressor gene is the p53 gene, discovered over 2 decades ago.

Normally, p53 acts as a regulating mechanism for cell division and alterations in the cell DNA are associated with rapid increases in cellular content of this protein (Levine 1997). There is increased p53 gene activity to exert its function as a transcription factor when triggered by cellular stresses as in oncogene activation, DNA damage, and hypoxia. This results in a cascade of events that eventually prevent tumour development (Nelson and Kastan 1994; Graeber et al. 1996)**.** Possibly because of its pivotal function, the *p53* gene is the most frequently altered gene in human cancers occurring in up to half of all human cancers and in approximately 20%–30% of breast cancers (Hollstein et al. 1996). Because *p53* mutations result in increased protein stability, over-expression of p53 has been used as a surrogate of *p53* dysfunction

(Osborne 2004). Host immune response produces antibodies (p53 autoantibodies) against the abnormally expressed p53 protein, which is also detectable relatively early during carcinogenesis (Labrecque 1993).

The association between serum p53 protein (Wu et al. 2010) and p53 autoantibodies (Lenner et al. 1999, Metcalfe 2000, Wu et al. 2010) expression and the risk of breast cancer has been studied. These studies, however, are limited by the lack of the longitudinal quality and potential population impact of the risk estimates because of the inclusion of already diagnosed breast cancer cases as the study population. According to our knowledge, studies investigating the association between prediagnostic serum p53 protein and p53 autoantibody levels with subsequent risk of pregnancy-associated breast (PABC) cancer do not exist.

5.5   Possible joint hypotheses

Apart from understanding the role of individual biomarkers on the risk of PABC, it is likely that an interaction between these factors leads to a joint functional effect. Vitamin D, for instance, is immunosuppressive and has been associated with various autoimmune diseases (Cantorna et al. 1996, Ascherio et al 2010). It is possible that the EBV infection or reactivation may be altered by vitamin D levels, with a consequent effect on the risk of breast cancer occurring soon after pregnancy. This is especially important at pregnancy, which is thought to be an immune-suppressive window of life, during which a mother's immune response is tapered to accept her fetus.

Animal studies have also shown that p53 null mice are more susceptible to development of breast cancer when inoculated with endogenous hormones which increase proliferation (Rajkumar et al. 2007). It is likely that individuals expressing p53 autoantibodies (an indication of dysfunctional p53) at different levels of endogenous hormones may show varied risk of PABC.

Also, vitamin D status in humans modulates the immune response to and reactivation of EBV (Tsoukas et al. 1984), and interplay of the two has recently been suggested to play a role in chronic disease (multiple sclerosis) development (Holmøy 2008), often following pregnancy. It is possible that the association between EBV and PABC is

determined by the levels of vitamin D. The present study utilises molecular epidemiological tools to evaluate the role of prediagnostic biomarkers on the risk of development of a more aggressive sub-group of breast cancer, pregnancy-associated breast cancer (Breast cancer occurring during pregnancy or one year post-partum).

6 Aims of the study

To better understand the multi-factorial etiology of pregnancy related breast cancer, the present study evaluates to the role of prediagnostic serum biomarkers as risk markers of the subsequent development of breast cancer, and pregnancy-associated breast cancer (PABC). The specific aims of this study were to study:

1. The effect of the duration of storage and season of sample withdrawal on the serum levels of vitamin D and androstenedione

2. The association between prediagnostic serum vitamin D levels and risk of breast cancer, and PABC

3. The association between past Epstein-Barr virus infection and reactivation on the risk of subsequent development of PABC

4. The association between prediagnostic p53 protein and p53 autoantibodies, and the joint effects with vitamin D and androstenedione on risk of PABC

7   Material and Method

7.1   Serum biobank

A biological specimen bank, also called a biobank, is "a system which stores one or many types of biological specimens for later analysis from single or multiple studies under conditions which permit efficient retrieval and optimum stability of the samples" (Winn et al. 1990). The biological specimen bank captures and stores a specific moment in the life of the study participants, in the same way that an archived completed health questionnaire or a stored radiograph does. This is especially important in longitudinal studies, whereby an understanding of temporal sequencing of events is of interest.

In Finland there are a number of decades-old biobanks which mainly differ in their coverage, the type of material archived and the sampled participants. These biobanks are linkable by unique personal identification data (PID) to comprehensive population-based health registries, thus providing possibilities for studies with adequate statistical power for rare diseases and exposures (Pukkala et al. 2007). In the past ten years, informed consent has been collected from persons donating biological samples, with clear indication that the samples will be used for purposes of research. For older samples, enacted legislation or opt-out public broadcasts have formalized the use of the samples. The largest of these biobanks is the Finnish Maternity Cohort biobank.

7.2   The Finnish Maternity Cohort

The Finnish Maternity Cohort (FMC) was established in 1983. With a national coverage of approximately ninety-eight percent in Finland (Pukkala et al. 2007) it has over 1.6 million serum samples collected at first trimester of pregnancy. The samples, collected primarily for the screening of congenital infections and rubella, are withdrawn at municipal maternity care units at gestational weeks 8 to 12, following an informed consent. Thereafter, a volume of serum (1-3 ml) is stored at -25 ^{0}C in polypropylene cryo vials at the National Institute for Health and Welfare (previously National Public

Health Institute), Oulu, Finland (Koskela et al. 2000). Data on reproductive or medical history, personal identification data (PID), and demographic variables are available in the data files of the biobank. Storage of the serum samples is for the primary purpose of epidemiological research.

7.3   Finnish Cancer Registry

The Finnish Cancer Registry (FCR), the national population-based cancer register in Finland, is a population-based cancer registry which collects data on all new cases of cancer occurring in a well-defined population. All cancer cases diagnosed in Finland since 1953 are reported to the FCR, and coverage of the national cancer registry is virtually complete (>98%) with no loss to follow-up (Teppo et al 1994). The National Board of Health requests all physicians, hospitals and pathology laboratories to notify the FCR of diagnosed cases of cancer. This notification was made mandatory since 1961 in Finland.

All diagnosed cancer cases are sent to the FCR in electronic (online) or paper format. Information sent is immediately stored in the database of the registry. Visual and automatic checking procedures for illogical order of data entry dates or erroneous code combinations are applied. For notifications with incomplete or controversial data, further request for clarification is sent to the physician, hospital or laboratory in question (Teppo et al. 1994). At the FCR, case files are regularly linked with the Finnish population-based register to check the correctness of PID, and to obtain other relevant data such as the complete name, vital status, place of residence at the time of diagnosis and possible date of emigration. The registry is highly regarded by both the scientific and the healthcare community in Finland, and has been widely used in research, administration and education at different levels.

For research purposes, permission for linkage information between the FMC, the FCR and population registers are obtained from the Finnish national data protection authorities and the local ethical committees. Identification of individuals who participate in sample donation is prohibited but samples can be used for research purposes, based on participants' consented information.

7.4 Quality assurance

The quality of molecular epidemiologic research based on a long term archive of biological material depends on the existence of appropriately standardized protocols for the collection, processing and archiving of the samples. Variability in these stages may introduce systematic differences in the samples (Landi and Caporaso 1997). As biobanks accrue relevant specimens and potential for stored specimens in medical research becomes more important, the quality of the decades-old archived biological data is cardinal. Since the stability of biobank serum samples may be affected by the conditions of storage such as temperature, desiccation, contamination, and storage duration, there is need for scientific understanding of the stability of some biomarkers.

7.5 Nested case-control design

In the past few years, case-control studies nested within a prospective cohort have played a key role in understanding new risk factors for rare chronic diseases such as breast cancer. In a nested case-control study, the cases (upon diagnosis) that occur in a defined cohort are identified and, for each, a specified number of matched controls is selected from among those in the cohort who have not developed the disease by the time of disease occurrence in the case (Ernster 1994). In molecular epidemiology, it constitutes the collection and storage (freezing) of samples from participants at entry (cohort). The defined cohort is then followed up until a sufficient number of diseased individuals (cases) are identified (Lajous and Zhang 2010). Exposure assessment and other vital information are thereafter retrieved from the stored sample biobank and registry files for cases and controls that constitute a sub-sample of disease-free individuals (Silva IS 1999b). The popularity of the nested case-control study in recent years may be attributed to its advantages in that it is relatively cheaper (as it usually requires a smaller sample size), once the cohort has been established. Also, there is no potential bias due to loss to follow-up as it is based on a prospectively established cohort. The nested case-control study design used in this study is made possible because of the availability of the decades-old FMC, and the FCR.

Permissions for linkage to carry out this study were obtained from the Finnish data protection authorities and ethical review boards of the FMC and the University of Tampere.

7.6 Study population

In study I, the population was a random sample of women having been pregnant between 1984 and 2002. In the second study, the two groups of cases were included: women who developed breast cancer during pregnancy or one year thereafter (PABC) as well as women whose cancer was diagnosed over a year after pregnancy but not more than ten years. The study population consisted of singleton pregnancies, with cases and controls differing only by breast cancer diagnosis status. A summary of the demographic data is illustrated in **Table 1a** below.

Table 1a- Baseline characteristics of a stratified, random sample of women stemming from the Finnish Maternity Cohort with single and paired pregnancy samples to study the effect of storage time (up to 24years) on vitamin D and steroid hormone stability (study I).

	1st pregnancy samples		1st and 2nd pregnancy samples	
	winter	summer	winter	summer
Number (N)	163.0	161.0	38.0	40.0
Mean age (years)	28.6	28.7	30.8	31.2
Mean storage time (years)	14.9	14.9	13.2	12.3

7.6.1 *Study I*

The samples for this study were randomly selected based on samples in a previous study (Holl et al. 2008). The authors retrieved 10 randomly selected first pregnancy samples for every other year from 1984 to 2002 (totalling 100 samples). Four comparable first pregnancy samples were selected for our study taking into account the sampling season (two samples for the same season (summer) and two for the opposite season (winter)), age (+/- 4 years) and municipality or residence. Eventually, 402 samples were selected, making 201 each for summer samples winter. We also randomly selected 40 such individuals whose first and second pregnancies were from the same season (summer/summer and winter/winter) and 40 such individuals whose first and second pregnancy samples were from different seasons (summer/winter and winter/summer). Complete paired sample sets were available for 74 individuals. Season of sample collection was defined as winter (late December to early March) and summer (May to August).

7.6.2 *Studies II, III and IV*

For the breast cancer-related studies, risk estimates were obtained by conducting case-control studies nested within the Finnish Maternity Cohort.

In study II, to evaluate the association between vitamin D and breast cancer, there were two sets of breast cancer cases:

- 100 breast cancer cases diagnosed within the first five years of sample withdrawal for the first pregnancy and 10 years for the subsequent pregnancy (Henceforth called non-PABC samples for clarity).

- 111 PABC cases. The prospectively sampled PABC cases were defined as women diagnosed within one year from date of sample withdrawal.

Controls were matched by incidence density sampled cases on a one-to-one ratio for age (+/- 1 year), year and season of sample withdrawal, parity (+/- 1, number of pregnancies at the time of sample withdrawal).

For the studies III and IV, a second set of controls were matched (same matching criteria as above) to the previously sampled PABC cases. This was done to increase power to evaluate joint effects. PABC cases were sampled from 1986 to 2005, with the highest number (10) in 1990 and the lowest (1) in 1987.

Table 1b- Baseline characteristics of cases and controls for serial and PABC[1] sample population of the Finnish Maternity Cohort (study II to IV). For studies III and IV, a second set of controls matched to the PABC cases was identified.

	1st pregnancy samples		2nd pregnancy samples		[1]PABC	
	Cases	Controls	Cases	Controls	Cases	Control
Number (N)	100	100	100	100	111	111
Median age[2]	30.3	30.4	33.1	33.4	34.7	34.8
Gestational day (days)	73.4	73.5	78.7	77.1	77.9	
Follow-up time[2]	7.4		4.6		1.0	
Age at diagnosis[2]	37.9		37.8		35.6	

[1] Pregnancy associated breast cancer (Breast cancer diagnosed within 1 year of sampling). A second control was selected for studies III and IV, making one-to-two case-control pairing.

[2] Mean lag-time (time from sample withdrawal to diagnosis) in years.

7.7   Laboratory Methods

All samples were analysed masked, with coded case-control status. Case-control pairs or triplets were analysed together in the same run, to minimize variation. Except for EBV Zebra IgG antibodies detection which was evaluated by an in house-made peptide-based Enzyme-Linked Immunosorbent Assay (ELISA), measurements were performed using commercial RIA or ELISAs as listed in **Table 2.**

Table 2. Summary of the laboratory methods

Analyte	Laboratory Method
25-OHD	IDS-RIA, IDS Ltd. Boldon, UK
EBV EBNA EA and VCA IgG antibodies	ELISAs, Biotest AG, Frankfurt, Germany
p53 protein	p53 ELISA, DIACLONE, Sweden
p53 autoantibodies	ELISA Dianova Hamburg, Germany
Androstenedione	DPC Immulite 2000 Androstenedione Chemiluminescent EIA, UK

EBV, Epstein-Barr virus

EBNA, Epstein-Barr virus nuclear antigen

EA, Early antigen

VCA, Viral capsid antigen

ELISA, Enzyme-linked immunosorbent assay

EIA, Enzyme immunoassay

Serum 25-OHD was quantified using a 25-OHD IDS-radioimmunoassay (RIA) from IDS Ltd, Boldon, UK. 160 µL aliquots of previously unthawed samples were sent to the Department of Anatomy at the University of Tampere, Finland, for laboratory analysis. The procedure entails two stages; the extraction phase and the quantification phase.

To quantify the serum levels of androstenedione, 160 µL aliquots of previously unthawed samples were also sent to the Department of Medical Biosciences, University of Umeå, Umeå, Sweden for laboratory analysis by competitive chemiluminescent enzyme immunoassay (DPC Immulite 2000 Androstenedione Chemiluminescent EIA, UK). With control sera, the inter-assay coefficient of variation (CV) was 16.9% at 2.2 ng/mL and 6.2% at 5.4 ng/mL.

The serum p53 protein and p53 autoantibodies were detected using commercially obtained ELISA from DIACLONE (DIACLONE, Sweden). The ELISA measures both wild-type and recombinant p53 proteins applying plates pre-coated with a monoclonal antibody specific to p53 protein. Standards with known p53 concentrations were used on all wells. A sample volume of 100 µl is required for the assay. Quantification of serum p53 autoantibodies was done using a commercial p53-autoantibody ELISA kit from Dianova (DIANOVA p53-autoantibodies ELISA Kit, Hamburg, Germany). Step Procedures were performed as directed by the manufacturer. The absorbance, which is directly proportional to the concentration of p53 protein and p53 autoantibodies levels were read at 450nm on a spectrophotometer, and quantification of p53 protein and p53 autoantibodies was extrapolated from a calibration curve.

Immunoglobulin G (Ig G) antibodies to EBV nuclear antigen (EBNA) and early antigen (EA) were assessed by commercial ELISAs based on recombinant proteins (Biotest AG, Frankfurt, Germany). The measurements were performed at the *Centro di Riferimento Oncologico, Istituto Di Ricovero e Cura a Carattere Scientifico,* Italy. Following the manufacturer's recommendations, a priori cut-off values were defined relative to internal positive and negative reference sera to define positive or negative samples on all plates. Samples that were EBNA IgG negative were also assessed for the viral capsid antigen (VCA) IgG to evaluate past infection. In addition, EBV Zebra

IgG antibodies were evaluated by an in house-made peptide-based ELISA, as previously described.

7.8 Statistical analysis

We used SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL) for all statistical analysis. A two-sided p value < 0.05 was considered statistically significant for all tests.

For study I, logarithmic transformation was performed on the serum 25-OHD levels because they were not normally distributed. Pearson's partial correlation coefficient (r) was used to test for correlation between duration of sample storage and both 25-OHD and androstenedione levels. Independent sample t-test was used to assess the effect of sampling season on single hormone measurement, and the paired sample t-test to assess the effect of the sampling season on paired (serial) hormone measurements.

For study II, quintile cut-off points were determined using 25-OHD quintiles levels of the controls. Relative risk estimates (expressed as odds ratio (OR)) at 95% confidence intervals (CI) of breast cancer were obtained by comparing quintile levels of serum 25-OHD, using Conditional Logistic Regression, with the lowest quintile as the reference. Separate analysis were performed for the first and second pregnancy levels (non-PABC), and PABC levels of 25-OHD. The breast cancer risk was also evaluated for women with clinically sufficient (≥ 75nmol/L) levels of serum 25-OHD (Holick 2007).

For the study III, ORs with 95% CI were estimated by conditional logistic regression for the matched case-control triplets, with EBV seronegative samples as the reference. The levels of serum 25-OHD were also categorized based on a previous definition of vitamin D sufficiency (Holick 2007), resulting in a digression of the case-control triplets. Thus, unconditional logistic regression was used to obtain relative risk estimates for the serological EBV markers and PABC risk, adjusted for the originally matched criteria.

For the study IV, group percentages were calculated and compared using the non-parametric Mann-Whitney test. Spearman's ranked correlation (r_s) was used to assess association between serum p53 protein and p53 autoantibody levels. OR with 95% CI for PABC were also derived by conditional logistic regression for matched case-control sets by levels of p53 protein and p53 autoantibodies (lowest level as reference). Likewise, for the joint effect, non-conditional logistic regression was used to obtain risk estimates for higher levels of p53 protein and p53 autoantibodies within categories (median level for controls) of vitamin D and androstenedione.

8 Results and comments

8.1   Study I – Quality control of FMC serum samples

- Storage of serum samples in the FMC biobank at -25 ^{0}C for up to 24 years does not affect 25-OHD detectability
- Observed significant difference in seasonal variation affects serial sample pairing.

There was no significant correlation between serum 25-OHD levels and storage time neither for the first pregnancy samples (r_s= -0.08) nor for the second pregnancy samples (r_s = -0.09). Similar results were obtained for androstenedione levels (r_s = 0.09).

There was a highly significant mean difference in the levels of 25-OHD for paired serum samples in two consecutive pregnancies during winter (first sample) vs. during summer (second sample) (mean difference $\mu_{d,}$= -18.4nmol/L; p-value ≤0.001, **Table 3a**). This was true (albeit to the opposite direction) also for the first samples withdrawn during summer versus the second samples withdrawn during winter (μ_d= 9.4nmol/L; p-value <0.1). Comparing the average serum 25-OHD levels for groups of individuals with different kind of pairs of sampling seasons (identical or opposite seasons), we found significant differences when winter-winter samples were compared to all other types of sample pairs **(Table 3b**). The highest differences were observed when comparing the average serum 25-OHD levels of winter-winter samples with summer-summer (μ_d= 16.6nmol/L, p-value ≤0.001) and summer-winter samples (μ_d= 17.8nmol/L; p-value ≤ 0.001). No statistically significant differences were observed when similar comparisons were made for androstenedione levels.

Table 3a Average differences in serum 25-OHD (nmol/l) in paired sera of pregnant Finnish women by season of sample withdrawal

Seasons of sample withdrawal	Mean differences In 1st and 2nd samples	P value
winter-summer(w-s)	-18.4	≤ 0.001
summer-winter(s-w)	9.4	0.10
summer-summer(s-s)	-1.9	0.67
winter-winter(w-w)	-5.1	0.07

Table 3b Differences in average serum 25-OHD (nmol/l) of paired pregnancy samples of Finnish women with specific pregnancy/sampling history

	Summer - Winter	Winter – Summer	Winter - Winter
	Mean difference[1] (p value)		
Summer- Summer	1.20 (0.83)	- 6.90 (0.11)	16.60 (0.001)[2]
Summer - Winter		- 8.04 (0.11)	17.80 (0.001)[2]
Winter - Summer			9.70 (0.02)[2]

[1]Mean differences between averages of paired serum

[2]Individual paired samples of 25-OHD for winter-winter pairs are significantly lower than all other individual season pairs

8.2 Study II – Prediagnostic vitamin D status and the risk of PABC

- Higher levels of serum 25-OHD were associated with increased risk of PABC, but not with non-pregnancy associated breast cancer.

In general, no statistical significant association of serum 25-OHD levels with the risk of breast cancer was observed quintile by quintile, or between the lowest versus other (2nd to 5th) quintiles combined, neither for the first (OR=1.4, 95%CI 0.6-3.4) nor for the second pregnancy samples (OR=1.4, 95%CI 0.7-2.9), **Table 4**). On the contrary, higher levels of vitamin D (2nd to 5th quintiles combined) were associated with an increased risk of PABC (OR=2.7, 95%CI 1.04 - 6.8). The OR was two to four times higher in the different quintiles of vitamin D levels compared to the lowest quintile (**Table 4**).

Table 4- Odds ratio (OR) with 95% confidence interval, CI) of breast cancer and pregnancy-associated breast cancer (PABC) by quintiles of serum vitamin D concentrations (Study II)

Quintiles	First Pregnancy OR (95% CI)	Second pregnancy OR (95% CI)	PABC OR (95% CI)
Q_2 vs Q1	1.5(0.5 - 2.0)	1.0(0.4 – 2.6)	2.9(1.0 - 8.4)
Q_3 vs Q1	1.2(0.4 - 3.6)	1.7(0.7 - 4.2)	2.0(0.7 - 6.0)
Q_4 vs Q1	1.2(0.4 - 3.7)	0.7(0.2 - 1.9)	3.7(1.2 - 11.8)
Q_5 vs Q1	1.4(0.5 - 4.2)	2.1(0.8 - 5.1)	1.9(0.5 - 6.7)
Q_{2-5} vs Q1	1.4(0.6 - 3.4)	1.4(0.7 - 2.8)	2.7(1.0 - 6.7)

Q_1 is the lowest (reference) quintile of serum 25-hydroxy vitamin D

8.3 Study III – Past EBV infection and the risk of PABC

- Whereas previous EBV infection was not associated with risk of PABC, EBV reactivation among individuals with more than sufficient levels of vitamin D was associated with increased risk of development of the disease.

The concentrations of anti-EA IgG and anti-ZEBRA IgG were significantly correlated (p < 0.04). There was no statistically significant association between the risk of PABC and antibodies to EBNA, EA or ZEBRA antigens (**Table 5a**). Individuals positive for either anti-EA IgG or anti-ZEBRA IgG antibodies had a somewhat increased risk of PABC with borderline statistical significance (OR= 1.6, 95% CI 1.0 – 2.8).

Table 5a   Risk estimates (OR with 95% CI) for pregnancy-associated breast cancer in relation to the detection of positive IgG antibodies to EBV antigens in the Finnish Maternity Cohort (Study III)

| | Number of | | | |
Seropositivity	Cases	Controls	ORs	95% CI
EBNA				
-ve	6	8	1	
+ve	102	200	0.7	0.2 – 2.2
EA				
-ve	30	73	1	
+ve	78	135	1.5	0.9 – 2.5
ZEBRA				
-ve	94	191	1	
+ve	14	17	1.4	0.7 – 2.8

Among individuals with more than sufficient levels of serum 25-OHD (≥ 75nmol/l), a highly increased risk of PABC was associated with positivity for serum EA IgG antibodies (OR= 7.7, 95% CI 1.4 – 42.3) and ZEBRA IgG antibodies (OR= 7.8, 95% CI 1.1 – 61.1). Furthermore among these women, positivity for either EA or ZEBRA IgG antibodies was significantly associated with the risk of PABC (OR= 12.1 95% CI 1.3 – 107.3). No such differences were found among individuals positive for EBV EBNA IgG antibodies (**Table 5b**).

Table 5b OR with 95% CI for pregnancy-asscociated breast cancer and EBV EBNA, EA and ZEBRA IgG antibody positivity among women with sufficient levels of vitamin D in the first trimester serum samples of the Finnish Maternity Cohort (Study III)

Seropositivity		Number of		OR[1]	95% CI
		Cases	Controls		
	Vit D (≥75 nmol/l)				
EBNA	-ve	0	0		
EBNA	+ve	13.0	24.0	n.a	
EA	-ve	2.0	14.0	1.0	
EA	+ve	11.0	10.0	7.7	1.4 – 42.3
ZEBRA	-ve	9.0	22.0	1.0	
ZEBRA	+ve	4.0	2.0	7.8	1.1 – 61.2

Vitamin D levels defined as sufficient (serum 25-OHD≥ 75 nmol/l)

[1]Adjusted for age and season of sampling

n.a = not available

8.4   Study IV – p53 expression and the risk of PABC

- Higher p53 autoantibody levels, but not p53 protein levels, tended to be associated with risk of development of PABC. The risk was significantly higher for women with higher levels of vitamin D and androstenedione.

Serum p53 protein concentrations were not correlated (r_s= 0.05, p< 0.4) with p53 autoantibody levels. Higher levels of serum p53 protein were not associated with the risk of PABC, whereas increased p53 autoantibody levels tended to be associated with risk of development of the disease (OR= 2.3, 95% CI 0.9 – 5.5, for highest versus lowest quintile) (**Table 6a**). This was even more so for women with vitamin D levels above the median among whom increased p53 autoantibody levels showed very high, albeit not significant, point estimates for the risk of PABC (OR=6.6, 95%CI 0.7 - 65.4) (**Table 6b**).

Table 6a OR with 95% CI of pregnancy-associated breast cancer for p53 proteins, p53 autoantobody and androstenedione levels in a Finnish Maternity Cohort sub-sample (Study IV).

	Cases	Controls	OR	95% CI
p53 protein				
0 – < 63	70	128	1.0	
63– <179	34	83	0.7	0.4 – 1.3
≥ 179	5	9	1.0	0.3 – 3.1
p53 autoantibody				
0 – < 35	85	187	1.0	
36 – < 67	16	28	1.2	0.6 – 2.5
≥ 67	10	9	2.3	0.9 – 5.5

Among women with levels of circulating androstenedione below the median, no association was found between p53 protein or 53 autoantibodies and the risk of PABC (Results not shown). In individuals with above median levels of androstenedione, increased p53 autoantibody levels were associated with a significantly increased risk of PABC (highest versus lowest category, OR=5.6, 95%CI 1.0 - 32.0).

Table 6b Risk estimates (OR with 95% CI) of pregnancy-associated breast cancer for joint effects of p53 proteins, p53 autoantobodies and androstenedione in different vitamin D strata of a Finnish Maternity Cohort sub-sample (Study IV).

Seropositivity	Cases	Controls	OR	95% CI[a]
[1]**Vit D (< 37 nmol/L)**				
p53 protein				
0 – < 63	34	63	1.0	
63 – <179	12	37	0.6	0.3 – 1.3
≥ 179	3	1	5.6	0.6 – 55.6
p53 autoantibody				
0 – < 36	40	81	1.0	
36 – < 67	4	12	0.7	0.2 – 2.2
≥ 67	5	6	1.7	0.5 – 5.9
[1]**Vit D (≥ 37 nmol/L)**				
p53 protein				
0 – 62	35	61	1.0	
64 – 178	21	42	0.9	0.5 – 1.7
≥ 179	2	8	0.4	0.1 – 2.2
p53 autoantibody				
0 – < 36	44	97	1.0	
36 – < 67	11	11	2.2	0.9 – 5.5
≥ 67	3	1	6.6	0.7 – 65.4

[1]Median level of serum 25-OHD (37 nmol/L)

9 Discussions

This study was conducted to evaluate prediagnostic vitamin D status, EBV infection and reactivation, p53 protein and p53 autoantibodies as risk indicators for the development of breast cancer occurring during pregnancy. Considering the relatively small lag time between serum sampling and development of this disease, the strength of the longitudinal FMC biobank provides a unique opportunity to evaluate these associations. The effect of duration of storage on the stability of biomarkers, a factor of significant importance in molecular epidemiological studies, was first evaluated.

No variation was noted for serum vitamin D and androstenedione in serum samples of the FMC following storage of up to twenty-four years. Thus, serum samples stored at -25 ^{O}C can be used to study vitamin D- and androstenedione-disease associations. For vitamin D-disease related studies, the study findings, however, highlighted the importance of matching for the season of sample withdrawal, for single samples as well as serial sample measurements.

Prediagnostic circulating vitamin D levels at the first and second pregnancy, independently, were not associated with the risk of breast cancer in general. On the other hand, higher levels of vitamin D were associated with the risk of development of PABC.

Past EBV infection was not associated with risk of development of PABC whereas EBV reactivation amongst individuals with more than sufficient levels of vitamin D was associated with a highly significantly increased risk of the disease.

Whereas higher serum p53 autoantibodies tended to be associated with the risk of PABC, serum p53 protein was not associated with risk of PABC. Among women with more than the median levels of vitamin D and androstenedione, higher levels of p53 autoantibodies were highly associated with increased risk of development of PABC.

9.1 Comparison of the study results to other studies

Despite evidence that the incidence of PABC is increasing, studies on the disease are scarce, and provide a limited scope for comparison. However, comparison to breast cancer in general is valid because of their more or less common aetiology.

9.1.1 *Association between serum 25-hydroxyvitamin D and pregnancy-associated breast cancer*

Evidence from laboratory studies implicating higher vitamin D levels to reduced risk of breast cancer is based on the fact that the more active form, 1, 25-$(OH)_2D$, inhibits cell proliferation and induces differentiation and apoptosis in normal and malignant breast cells (Colston and Hansen 2002, Welsh et al. 2003, Lowe et al. 2003). Later findings have, however, shown that the more stable form, 25-hydroxyvitamin D, also has some hormonal properties (Lou et al. 2004) and protects against cellular stress in breast epithelial cells (Peng et al. 2010). The role of 25-OHD in the aetiology of breast cancer is therefore not limited to the indirect action via 1, 25-$(OH)_2D$, but also to its direct action as a hormone.

The present study is the first, to our knowledge, to evaluate the association between prediagnostic serum levels of 25-OHD levels and risk of development of breast cancer occurring during or soon after pregnancy. Only 12% of our study population had sufficient levels of vitamin D, consistent with the high prevalence of vitamin D insufficiency in pregnant women populations (Kazemi et al. 2009, Yu et al. 2009).

We found no association between serum 25-OHD levels and the risk of breast cancer among the non-PABC cases. The result is consistent with findings in prospective studies among postmenopausal women (Bertone-Johnson et al. 2005, Freedman et al. 2008, Chlebowski et al. 2008, McCullough et al. 2009), a sub-group of premenopausal women (Bertone-Johnson et al. 2005) and in two recent meta-analyses (Gandini et al. 2010, Yin et al. 2010). In a more recent case-control study nested within The *Malmö Diet and Cancer Study* (Almquist et al. 2010), a weak non-significant inverse

association was noted among postmenopausal women, and a non-significant positive association for a subset of premenopausal women.

Despite the strong experimental evidence supporting the anti-carcinogenic effects of vitamin D, our findings in the non-PABC population, together with the observed inconsistent findings in other longitudinal observational studies are probably due to variations in the study population. Moreover, the timing of the onset of cancer where vitamin D levels may be crucial is unknown. Unlike previous studies, the present study considered two measurements of prediagnostic serum vitamin D levels with a lag of 5 years. Further studies which utilise more than two serial sample measurements of vitamin D levels may not provide more insight into the role of vitamin D in breast cancer unless the follow-up is much longer.

Intriguingly, this study observed that higher levels of prediagnostic serum 25-OHD levels were associated with a significantly increased risk of development of PABC, the more fatal sub-group of breast cancer occurring among premenopausal women.

The positive association between levels of serum 25-OHD and the risk of PABC, though in contrast with evidence from animal studies (Colston an Hansen 2002, Welsh et al. 2003, Lowe et al. 2003), supports that higher circulating levels of serum 25-OHD are positively associated with elevated risk of other aggressive cancer forms such as aggressive prostate cancer (Tuohimaa et al. 2004, Ahn et al. 2008) and pancreatic cancer (Stolzenberg-Solomon et al. 2009, Stolzenberg-Solomon et al. 2010).

The mechanism underlying the positive association between vitamin D and risk of PABC is unclear. Levels of vitamin D binding protein (VDBP) are significantly increased during pregnancy (Haddad 1995). VDBP is the carrier protein of vitamin D in circulation, with higher affinity for 25-OHD than for 1, $25\text{-}(OH)_2D$ (Bouillon et al. 1981), probably increasing the relative availability of the biologically more active, anti-carcinogenic form, 1, $25\text{-}(OH)_2D$. Our observation might be explained by an inverse association between the free levels of the two forms of vitamin D. On the other hand, higher 25-OHD levels within the body may affect its metabolism, e.g., increased 24-hydroxylation (Welsh 2004), and lead to reduced tissue concentrations of 1, $25\text{-}(OH)_2 D$, with again consequent low anti-proliferative activity. It is also possible that the observation is due

to a pre-existing disease, considering the short follow-up time between sample withdrawal and diagnosis for the PABC cases.

9.1.2 *Association of Epstein-Barr virus and risk of pregnancy-associated breast cancer*

This is the first study, to our knowledge, to evaluate the interplay between prediagnostic EBV reactivation and vitamin D with the risk of PABC. Almost all individuals in this study showed EBV seropositivity, comparable to other results among pregnant women populations in Finland and Iceland (Lehtinen et al. 2003) as well as in the U.S.A (Haeri et al. 2010). The ubiquitous nature of the virus explains the high prevalence of EBV seropositivity observed. EBV reactivation occurs frequently during pregnancy (Costa et al. 1985, Haeri et al. 2010), probably due to reduced cellular immune response associated with pregnancy.

The null association between past EBV infection and risk for subsequent development of PABC in this study has been previously reported among premenopausal women (Richardson et al. 2004), with no association between positivity for EBV and risk of breast cancer. In another study (Joshi et al. 2009), the mean anti-EBNA IgG antibody levels were significantly higher in breast cancer cases than in controls with benign breast disease. Both studies, however, lack the temporality for risk association (the cause precedes the effect) since EBV seropositivity for cases were measured after cancer diagnosis. In a more recent case-control study nested within the Janus Serum Bank cohort (Cox et al. 2010), EBV IgG antibody levels in serum samples drawn at least four years before breast cancer diagnosis were not associated with the risk of the disease.

Methodological variation in detecting EBV in different studies has also been suggested to explain the differences in results (Murray 2006). In this study, we employed four serological markers of EBV infection, and found that EBV EA antigen antibodies reflecting the expression of EBV early antigens were notable in a proportion of PABC cases. The longitudinal and population-based nature of sample collection and cancer

registration minimize the possibilities that our results are due to reverse causality bias or chance introduced because of case mis-classication.

Positivity for both serum anti-EBV ZEBRA Ig G and anti-EBV EA IgG antibodies confirm occurrence of EBV reactivation which was notable almost exclusively in individuals with sufficient levels of vitamin D. These women, who showed positivity for anti-EBV EA or ZEBRA IgG antibodies had a significantly increased risk for PABC. These observations are reminiscent of hypotheses on other associations between EBV, vitamin D chronic diseases such as multiple schlerosis (Holmøy 2008, Ascherio et al. 2010). The active form of vitamin D, dihydroxyvitamin D_3, is a potent immunomodulator, and may modulate the immune response to EBV infection by suppressing T-cell proliferation (Lemire 2000, Holmøy 2008). It is possible that at higher levels of vitamin D, the immune surveillance to EBV is reduced, thus promulgating viral reactivation from EBV harbouring B cells.

The fact that EBV readily transforms normal human B cells into lymphoblastoid cells that grow perpetually, lent credence to its oncogenic nature, since immortal cell growth in culture is one of the major characteristics that differentiate cancer cells from normal cells (Kieff 2010). Upon EBV reactivation, the viral ZEBRA protein further down-regulates the immune response by inducing the expression of cytokines IL-10, a negative regulator of macrophages and NK cell functions (Moore et al. 1993). There is also evidence that EBV reactivation proteins elicit anti-apoptotic response through several pathways (Lin and Flemington 2010). In a laboratory study, Inman *et al.* (Inman et al. 2001) showed that upon in vitro activation, ZEBRA-negative EBV-positive cancer cells undergo apoptosis, whereas there was no apoptosis for the ZEBRA-positive population. The inhibition of apoptosis together with the altered immune response provides suitable conditions for the progression of transformed cells and/or an already existing cancer.

The observed association between EBV reactivation markers and risk of development of PABC among women with more than sufficient levels of vitamin D provides evidence for contemplating possible point effects of the two (EBV and vitamin D) in chronic disease associations (Holmøy 2008). The clinical implications of EBV reactivation in cancer epidemiology warrants further studies.

9.1.3 *Association of p53 protein and p53 autoantibodies and risk of pregnancy-associated breast cancer*

The observation that prediagnostic levels of p53 protein are not associated with the risk of PABC, though contrary to previous positive findings on benign breast disease (Rohan et al. 1998), is consistent with an earlier study which found no significant association between p53 accumulation in benign breast tumor biopsy specimens and risk of progression to breast cancer (Younes et al. 1995). The detection of p53 proteins in this study is based on over-expression and detection of free serum p53 protein. It is possible that the p53 proteins form complexes with p53 autoantibodies soon after their expression and are not identified in serum for long. This might also be due to the over expression of p53 protein antagonists, leading to its degradation. In tumours without any signs of mutation in the p53 gene, the human form of murine double minute 2 protein (HDM2), a p53 antagonist, is over-expressed fostering the premature degradation of p53 protein (Mori et al. 2004).

On the other hand, the somewhat positive association between p53 autoantibody levels and risk of PABC is in tandem with previous observations in a cross-sectional study (Lenner et al. 1999). In that study, the presence of high levels of p53 autoantibodies was associated with a 9-fold increased risk of breast cancer. The cross-sectional case-control setting comparing newly diagnosed breast cancer cases to cancer-free controls lacks the temporality to evaluate a causal association. Our study exploits a longitudinal setting by using prediagnostic serum samples and a modestly increased risk for the development of PABC in individuals positive for p53 autoantibody at the first trimester of pregnancy.

To the best of our knowledge, no study has reported associations between prediagnostic levels of p53 protein and p53 autoantibodies at pregnancy and the risk of PABC. The strength of our study lies on the prospective nature of the study, and the observed stability of the biomarkers in the serum samples of our study material (Study I).

9.2   Biobank-based approach in the breast cancer context

Biobank-based research has become increasingly important in recent years, despite the associated cost, because of the power of inference inherent in the prospective nature of the sample collection. Standardized processes entailing the collection of samples from participants up to storage for research purposes is critical to the quality of biobank-based research. Non-standardized alterations in the collection, handling and storage of the samples may have enormous consequences on the quality of the material, hence on the results and recommendations based on the analysis (Männistö et al. 2007). FMC samples are readily available for vitamin and hormone measurements.

The use of biobank data offers proper time order for which data is collected for the exposure and outcome, and decreases the possibility of "reverse causality bias", i.e. the mixing up of cause and effect (Pukkala et al. 2007). This is even more important in individuals with PABC who generally turn to present with a more fatal disease. For the samples were collected prior to the date of diagnosis, temporality can be conveniently assumed. The nested-case control design with incidence density sampling employed in these studies has the advantage that there is no need to follow-up the controls beyond case's diagnosis. Meanwhile matching reduces bias due to known confounders such as age at sample withdrawal.

9.3   Validity of the assays and the markers used

The measurement of serum 25-OHD over the more active 1, 25-$(OH)_2$D as a marker of vitamin D status is because 25-OHD is more stable and easily responds to subcutaneous production and vitamin D intake (Hollis 2008). Like for 1, 25-$(OH)_2$D , it has been observed that 25-OHD has hormonal properties (Lou et al. 2004) and protects against cellular stress in breast epithelial cells (Peng et al. 2010). Its role in the aetiology of breast cancer is therefore not limited to the indirect action via 1, 25-$(OH)_2$, but also to its direct action as a hormone. The commercial RIA for quantification of serum 25-OHD is selectively sensitive to the measurement of 25-OHD_3 over 25-OHD_2 (Hollis 2008) and the values quantified may represent an underestimate of the true levels of circulating vitamin D levels.

9.4   Sample size and generalisability of the results

To our knowledge, this is the first study to investigate the molecular indicators for risk of PABC. For breast cancer disease association, we had a modest sample size for the Study II. The power was increased in Studies II and III by selecting a second set of controls. The larger sample size increases the precision (reducing random error) in the estimates and therefore and our ability to reveal associations. The FMC and other Nordic maternity cohorts are shown to have good population representativeness, with no significant difference in cancer incidence as compared to their respective national rates (Pukkala et al. 2007). The findings of this study can therefore be generalized to all Finnish women. The findings from this study should, however, be interpreted with caution, particularly when compared to non-pregnant women populations. The homogeneous source population of this study reduces the generalisability to pregnant women populations of other ethnic groups.

9.5   Strengths and limitations of the study

The use of serum molecular indicators (biomarkers) as a measure of exposure to risk factors long before disease occurrence is a substantial advancement in epidemiological research. The use of self-reported questionnaires or interviews as surrogates of circulating biomarkers usually introduces differential misclassification such as recall bias. The advantage of measurement of serum biomarkers eliminates such biases.

Except for the disease status, the cases and controls in the study were quite comparable. All samples were pregnant (gestational weeks 8- 12) at sample withdrawal. Due to the effect of pregnancy-associated plasma volume expansion on biomarker estimation (Faupel-Badger et al. 2007), only women with singleton pregnancies were included in this study. The cases included only women with a primary diagnosis of breast cancer.

We observed no changes in the levels of serum vitamin D and androstenedione following years of freeze-storage. Ideally, the measurement of sample degradation over a long period of time should entail one measurement at collection time, followed by

several measurements over the duration of storage. A setback in this context is that the FMC biobank until recently did not take into account the number of freeze-thaw cycles per sample. Fortunately, serum 25-hydroxyvitamin D has been shown to be unaffected by up to 4 multiple freeze-thaw cycles (Antoniucci et al. 2005).

Although the present study did not evaluate the effect of storage time on immunological markers to EBV following serum sample storage, there is evidence that the antibodies to EBV are readily detectable and not affected by storage (Stevens et al. 2007). The follow-up time for PABC cases was one year, a relatively short time course for risk factor analysis in breast cancer etiology.

In this study, only parity as a reproductive risk factor for breast cancer was matched for among the cases and controls. The definition of parity in this study was limited to the number of pregnancies at time of serum sample withdrawal with no information for subsequent pregnancies. This might have resulted in confounding given that the pregnancies could have correlated with abortions which might increase breast cancer risk (Hajian-Tilaki and Kaveh-Ahangar 2010). All women were parous and the effect of additional pregnancies on the risk of breast cancer risk is modest (Wohlfahrt and Melbye 2001).

Confounding

Due to its prospective nature of this study, the results less likely to be influenced by selection bias or reverse causality, the mixing up of cause and effect. Thus, higher levels of a putative risk factor in cases than in controls is more convincing evidence that the cause-effect relation exists between the putative risk factor and the disease of interest. The prospective nature of data collection also provides opportunities for cases and controls to be matched for known confounding factors (Crea 2001).

Based on previous studies, we identified some potential confounders. Two strong confounders, age and parity were considered in the inclusion criteria. Age at first full-term pregnancy and age at menarche, notable covariates of breast cancer risk in premenopausal women (Clavel-Chapelon et al. 2002, Lagiou et al. 2003), were available but not considered in the analysis. The mechanism by which these factors

affect breast cancer risk is unclear. They are thought to be surrogate measures of the endogenous hormone exposure. The present study found no association for androstenedione levels and the risk of development of breast cancer occurring during or soon after pregnancy (Results not shown).

Due to the seasonal variability of vitamin D, the season of sample withdrawal was equally matched for among the individuals. Furthermore, endogenous vitamin D levels are less prone to misclassification (biasing results towards the null) compared to estimates such as vitamin D intake.

The possibility that the observed associations are due to chance or residual confounding cannot be ruled out. For the PABC, the time from sample withdrawal to breast cancer diagnosis is relatively short. Data on genetic markers of breast cancer susceptibility (e.g. BrCa 1 and 2), past oral contraceptive use and occupational exposures was not available. However, mutation in the BRCA 1/ 2 genes is rare among PABC cases (Johannsson et al. 1998, Siegelmann-Danieli et al. 2003).

10 Conclusions

The present study was carried to increase understanding on the etiology breast cancer occurring during pregnancy with the following conclusions:

1. Serum samples stored in the FMC biobank at -25 ^{0}C for up to 24 years can be used to study hormone and vitamin D-disease associations. It is important to appropriately match for season of sample withdrawal in the case of vitamin D.

2. Higher levels of vitamin D were associated with increased risk of breast cancer occurring during or soon after pregnancy. No such association was observed for breast cancer in general. The positive association between vitamin D and PABC is consistent with observations in other fatal cancers.

3. Previous EBV infection was not associated with increased risk of PABC, whereas serological EBV reactivation markers among individuals with more than sufficient levels of vitamin D were associated with significantly increased risk of PABC.

4. Higher levels of p53 autoantibody showed a modest increased risk for development of PABC, which was statistically significant among women with above median levels of vitamin D and androstenedione.

This is the first study to provide direct epidemiological evidence on important biomarkers of the risk of breast cancer occurring during or soon after pregnancy. The mechanisms underlying the observed association are largely unclear. Further studies are warranted to better understand the role and potential impact of circulating biomarkers on the risk of breast cancer occurring during or soon after pregnancy.

Acknowledgements

List of references

Aebi S, Loibl S (2008): Breast cancer during pregnancy: medical therapy and prognosis. Recent results Cancer Res 178: 45–55.

Ahn J, Peters U, Albanes D, Purdue MP, Abnet CC, Chatterjee N, Horst RL, Hollis BW, Huang WY, Shikany JM, Hayes RBProstate, Lung, Colorectal, and Ovarian Cancer Screening Trial Project Team (2008): Serum vitamin D concentration and prostate cancer risk: a nested case-control study. J Natl Cancer Inst 100:796-804

Alm El-Din MA, Hughes KS, Finkelstein DM, Betts KA, Yock TI, Tarbell NJ, Aisenberg AC, Taghian AG (2009): Breast cancer after treatment of Hodgkin's lymphoma: risk factors that really matter. Int J Radiat Oncol Biol Phys 73: 69-74

Almquist M, Bondeson AG, Bondeson L, Malm J, Manjer J (2010): Serum levels of vitamin D, PTH and calcium and breast cancer risk-a prospective nested case-control study. Int J Cancer 127: 2159 - 2168.

Al-oanzi ZH, Tuck SP, Raj N, Harrop JS, Summers GD, Cook DB, Francis RM, Datta HK (2006): Assessment of vitamin D status in male osteoporosis. Clin Chem 52:248–54

American Cancer Society: www.cancer.org

Anderson BO, Petrek JA, Byrd DR, Senie RT, Borgen PI (1996): Pregnancy influences breast cancer stage at diagnosis in women 30 years of age and younger. Ann Surg Oncol 3: 204-211

Andersson TM, Johansson AL, Hsieh CC, Cnattingius S, Lambe M (2009): Increasing incidence of pregnancy-associated breast cancer in Sweden. Obstet Gynecol 114: 568-572

Antoniucci DM, Black DM, Sellmeyer DE (2005): Serum 25-hydroxyvitamin D is unaffected by multiple freeze-thaw cycles. Clin Chem 51: 258-261

Arbach H, Viglasky V, Lefeu F, Guinebretière JM, Ramirez V, Bride N, Boualaga N, Bauchet T, Peyrat JP, Mathieu MC, Mourah S, Podgorniak MP, Seignerin JM, Takada K, Joab I (2006): Epstein–Barr virus (EBV) genome and expression in breast cancer

tissue: effect of EBV infection of breast cancer cells on resistance to paclitaxel (Taxol). *J. Virol* 80: 845–853

Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists' Group, Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M (2008): Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. Lancet Oncol 9: 45-53

Ascherio A, Munger KL, Simon KC (2010): Vitamin D and multiple sclerosis. Lancet Neurol 9: 599-612.

Barthelmes L, Davidson LA, Gaffney CH, Gateley CA (2005): Pregnancy and breast cancer. BMJ 330 :1375 –1377

Beadle BM, Woodward WA, Middleton LP, Tereffe W, Strom EA, Litton JK, Meric-Bernstam F, Theriault RL, Buchholz TA, Perkins GH (2009): The impact of pregnancy on breast cancer outcomes in women<or=35 years. Cancer 115:1174 - 1184.

Beasley JM, Coronado GD, Livaudais J, Angeles-Llerenas A, Ortega-Olvera C, Romieu I, Lazcano-Ponce E, Torres-Mejía G (2010): Alcohol and risk of breast cancer in Mexican women. Cancer Causes Control 21: 863- 870

Bensyl DM, Iuliano DA, Carter M, Santelli J, Gilbert BC (2005): Contraceptive use--United States and territories, Behavioral Risk Factor Surveillance System. MMWR Surveill Summ 54: 1-72

Bernstein L (2002): Epidemiology of endocrine-related risk factors for breast cancer. J Mammary Gland Biol Neoplasia 7: 3–15

Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, Mandelblatt JS, Yakovlev AY, Habbema JD, Feuer EJ (2005): Cancer Intervention and Surveillance Modeling Network (CISNET) Collaborators. Cancer Intervention and Surveillance Modeling Network (CISNET) Collaborators. Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 353:1784-1792

Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willet WC, Hankinson SE (2005): Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidemiol Biomarkers Prev 14: 1991-1997

Berube S, Diorio C & Brisson J (2008): Multivitamin–multimineral supplement use and mammographic breast density. Am J Clin Nutr 87: 1400–1404

Bittner JJ (1957): Recent studies on the mouse mammary tumor agent. Ann. NY Acad Sci 21: 636–648

Bodnar LM, Simhan HN, Powers RW, Frank MP, Cooperstein E, Roberts JM (2007): High prevalence of vitamin D insufficiency in black and white pregnant women residing in the northern United States and their neonates. J Nutr 137: 447-52.

Boice JD Jr, Preston D, Davis FG, Monson RR(1991): Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients in Massachusetts. Radiat Res 125: 214–222

Bonnet M, Guinebretiere JM, Kremmer E, Grunewald V, Benhamou E, Contesso G, Joab I (1999): Detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 91: 1376 – 1381

Bonnier P, Romain S, Dilhuydy JM, Bonichon F, Julien JP, Charpin C, Lejeune C, Martin PM, Piana L (1997): Influence of pregnancy on the outcome of breast cancer: a case-control study. Societe Francaise de Senologie et de Pathologie Mammaire Study Group. Int J Cancer 72: 720 - 727

Booth BW, Boulanger CA, Smith GH (2007): Alveolar progenitor cells develop in mouse mammary glands independent of pregnancy and lactation. J Cell Physiol 212 : 729-736

Botha JL, Bray F, Sankila R, Parkin DM (2003): Breast cancer incidence and mortality trends in 16 European countries. Eur J Cancer 39: 1718 - 1729

Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P (1981): Influence of the vitamin D-binding protein on the serum concentration of 1, 25-dihydroxyvitamin D3. Significance of the free 1, 25-dihydroxyvitamin D3 concentration. J Clin Invest 67: 589–596

Boyd NF, Jensen HM, Cooke G, Han HL (1992): Relationship between mammographic and histological risk factors for breast cancer. J Natl Cancer Inst 84:1170–1179

Boyd NF, Lockwood GA, Byng JW, Tritchler DL, Yaffe MJ (1998): Mammographic densities and breast cancer risk. Cancer Epidemiol Biomarkers Prev 7:1133-1144

Brennan SF, Cantwell MM, Cardwell CR, Velentzis LS, Woodside JV (2010): Dietary patterns and breast cancer risk: a systematic review and meta-analysis. Am J Clin Nutr ;91: 1294-1302

Britt KL, Kendrick H, Regan JL, Molyneux G, Magnay FA, Ashworth A, Smalley MJ (2009): Pregnancy in the mature adult mouse does not alter the proportion of mammary epithelial stem/progenitor cells. Breast Cancer Res 11: R20

Brody L, Biesecker BB (1998): Breast cancer susceptibility genes: BRCA1 and BRCA2. Medicine 77: 208–226

Cantorna MT, Hayes CE, DeLuca HF (1996): 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci USA 93: 7861– 7864

Cardonick E, Dougherty R, Grana G, Gilmandyar D, Ghaffar S, Usmani A (2010): Breast cancer during pregnancy: maternal and fetal outcomes. Cancer J 16: 76-82

Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML, Perou CM (2007): The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13*: 2329–2334*

Cariati M, Purushotham AD (2008): Stem cells and breast cancer. Histopathology 52: 99-107

Chang-Claude J, Andrieu N, Rookus M, Brohet R, Antoniou AC, Peock S, Davidson R, Izatt L, Cole T, Noguès C, Luporsi E, Huiart L, Hoogerbrugge N, Van Leeuwen FE, Osorio A, Eyfjord J, Radice P, Goldgar DE, Easton DF; Epidemiological Study of Familial Breast Cancer (EMBRACE); Gene Etude Prospective Sein Ovaire (GENEPSO); Genen Omgeving studie van de werkgroep Hereditiair Borstkanker Onderzoek Nederland (GEO-HEBON); International BRCA1/2 Carrier Cohort Study (IBCCS) collaborators group (2007): Age at menarche and menopause and breast cancer risk in the International BRCA1/2 Carrier Cohort Study. Cancer Epidemiol Biomarkers Prev 16: 740-746.

Chlebowski RT, Johnson KC, Kooperberg C, Pettinger M, Wactawski-Wende J, Rohan T, Rossouw J, Lane D, O'Sullivan MJ, Yasmeen S, Hiatt RA, Shikany JM, Vitolins M, Khandekar J, Hubbell FA, Women's Health Initiative Investigators (2008): Calcium plus vitamin D supplementation and the risk of breast cancer. J Natl Cancer Inst 100: 1581-1591.

Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM (2001): No significant association of Epstein-Barr virus infection with invasive breast carcinoma. Am J Pathol 159: 571 - 578

Clavel-Chapelon F, E3N-EPIC Group (2002): Differential effects of reproductive factors on the risk of pre- and postmenopausal breast cancer. Results from a large cohort of French women. Br J Cancer 86: 723-727.

Colditz GA, Stampfer MJ, Willett WC, Hennekens CH, Rosner B, Speizer FE (1990): Prospective study of estrogen replacement therapy and risk of breast cancer in postmenopausal women. JAMA 264: 1648–1653.

Collaborative Group on Hormonal Factors in Breast Cancer (1996): Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Lancet 347: 1713- 1727

Collaborative Group on Hormonal Factors in Breast Cancer (2002): Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. Br J Cancer 87: 1234-1245.

Collaborative Group on Hormonal Factors in Breast Cancer Familial breast cancer (2001): collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease. Lancet 358: 1389–1399

Colston KW, Hansen CM (2002): Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. Endocr Relat Cancer 9: 45–59

Costa S, Barrasso R, Terzano P, Zerbini M, Carpi C, Musiani M (1985): Detection of active Epstein-Barr virus infection in pregnant women. Eur J Clin Microbiol Infect Dis 4: 335–336

Cox B, Richardson A, Graham P, Gislefoss RE, Jellum E, Rollag H (2010): Breast cancer, cytomegalovirus and Epstein-Barr virus: a nested case-control study. Br J Cancer 102: 1665 – 1669

Crea F(2001): Role of nested case-control studies in the difficult quest for new coronary risk factors. Heart 85: 153-158

Cui X, Dai D, Tseng M, Shu X-O, Gao Y-T, Zheng W (2007): Dietary patterns and breast cancer risk in the Shanghai Breast Cancer Study. Cancer Epidemiol Biomarkers Prev 16: 1443-1448.

Dawodu A, Wagner CL (2007): Mother-child vitamin D deficiency: an international perspective. Arch Dis Child 92: 737-740

De Boland AR, Boland RL (1994): Non-genomic signal transduction pathway of vitamin D in muscle. Cell Signal 6: 717-724.

Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA (2007):Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13: 4429– 4434

Dorgan JF, Reichman ME, Judd JT, Brown C, Longcope C, Schatzkin A, Campbell WS, Franz C, Kahle L, Taylor PR (1994): The relation of reported alcohol ingestion to plasma levels of estrogens and androgens in premenopausal women (Maryland, United States). Cancer Causes Control 5: 53- 60.

Dorjgochoo T, Shrubsole MJ, Shu XO, Lu W, Ruan Z, Zheng Y, Cai H, Dai Q, Gu K, Gao YT, Zheng (2008): Vitamin supplement use and risk for breast cancer: the Shanghai Breast Cancer Study. Breast Cancer Res Treat 111: 269– 278

Early Breast Cancer Trialists' Collaborative Group (2005): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365: 1687–1717

Eliassen AH, Missmer SA, Tworoger SS, Spiegelman D, Barbieri RL, Dowsett M, Hankinson SE (2006): Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. J Natl Cancer Inst 98: 1406- 1415

Ernster VL (1994): Nested case-control studies. Prev Med 23: 587-590.

Esserman LJ, Shieh Y, Park JW, Ozanne EM (2007): A role for biomarkers in the screening and diagnosis of breast cancer in younger women. Expert Rev Mol Diagn 7: 533- 544.

Fairfield K and Stampfer M (2007): Vitamin and mineral supplements for cancer prevention: issues and evidence. Am J Clin Nutr 85: 289S-292S.

Faupel-Badger JM, Hsieh CC, Troisi R, Lagiou P, Potischman N (2007): Plasma volume expansion in pregnancy: implications for biomarkers in population studies. Cancer Epidemiol Biomarkers Prev 16: 1720-1723.

Fawzy S, Sallam M, Awad NM (2008): Detection of Epstein-Barr virus in breast carcinoma in Egyptian women. Clin Biochem 41: 486 – 492

FCR-Finnish Cancer Registry. www.fcr.fi

Fentiman IS, Fourquet A, Hortobagyi GN (2006): Male breast cancer, *Lancet* 367: 595– 604

Ferlay J, Bray F, Pisani P, ParkinDM, GLOBOCAN 2002 (2004): Cancer incidence, mortality and prevalence worldwide. IARC Cancer Base No. 5 Version 2.0. IARC Press, Lyon

Ferlay J, Héry C, Autier P and Sankaranarayanan R (2010): Global Burden of Breast Cancer in Breast Cancer Epidemiology : 1-19

Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N (2005): Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst 97: 1652-1662.

Freedman DM, Chang SC, Falk RT, Purdue MP, Huang WY, McCarty CA, Hollis BW, Graubard BI, Berg CD, Ziegler RG (2008): Serum levels of vitamin D metabolites and breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. Cancer Epidemiol Biomarkers Prev 17: 889- 894

Friedenreich CM, Howe GR, Miller AB, Jain MG (1993): A cohort study of alcohol consumption and risk of breast cancer. Am J Epidemiol 137: 512- 520.

Gail MH, Costantino JP, Bryant J, Croyle R, Freedman L, Helzlsouer K, Vogel V (1999): Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst.* 91: 1829- 1846.

Gandini S, Boniol M, Haukka J, Byrnes G, Cox B, Sneyd MJ, Mullie P, Autier P (2010): Meta-analysis of observational studies of serum 25-hydroxyvitamin D levels and colorectal, breast and prostate cancer and colorectal adenoma. Int J Cancer (In Press)

Garland F, Garland C, Gorham E, Young J Jr (1990): Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. Prev Med 19: 614–622

Gaudet MM, Britton JA, Kabat GC, Steck-Scott S, Eng SM, Teitelbaum SL, Terry MB, Neugut AI, Gammon MD (2004): Fruits, vegetables and micronutrients in relation to breast cancer modified by menopause and hormone receptor status. Cancer Epidemiol Biomarkers Prev 13: 1485–1494

Gescher A, Pastorino U, Plummer SM, Manson MM (1998): Suppression of tumour development by substances derived from the diet--mechanisms and clinical implications. Br J Clin Pharmacol 45: 1-12

GLOBOCAN 2008. Cancer incidence, mortality and prevalence worldwide. IARC Cancer http://globocan.iarc.fr/

Gorham ED, Garland FC, Garland CF (1990): Sunlight and breast cancer incidence in the USSR. Int J Epidemiol 19: 820–824.

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379: 88–91.

Grant WB (2003). Ecologic studies of solar UV-B radiation and cancer mortality rates. *Recent Results Cancer Res*164: 371–377.

Greenle RT, Murray T, Boldin S, Wingo P (2000): Cancer statistics 2000. CA Cancer J Clin 50 :7–23.

Griffin BE (2000): Epstein-Barr virus (EBV) and Human disease: facts, opinions and problems. Mutat Res 462: 395 – 405

Haddad, J.G (1995): Plasma vitamin D-binding protein (Gc-globulin): multiple tasks. J. Steroid Biochem. Mol Biol *53*: 1–6.

Haeri S, Baker AM, Boggess KA (2010): Prevalence of Epstein-BarrVirus Reactivation in Pregnancy. Am J Perinatol 27: 715 – 719.

Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, Harris L, Hait W, Toppmeyer D (2006): Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. J Clin Oncol *24*: *5652*–5657.

Hahn KM, Johnson PH, Gordon N, Kuerer H, Middleton L, Ramirez M, Yang W, Perkins G, Hortobagyi GN, Theriault RL (2006): Treatment of pregnant breast cancer patients and outcomes of children exposed to chemotherapy in utero. Cancer107: 1219- 1226.

Hahn WC, Weinberg RA. Rules for making human tumor cells (2002): N Engl J Med 347: 1593-1603

Hajian-Tilaki KO, Kaveh-Ahangar T(2010): Reproductive factors associated with breast cancer risk in northern Iran. Med Oncol (In press).

Hance KW, Anderson WF, Devesa SS, Young HA, Levine PH (2005): Trends in inflammatory breast carcinoma incidence and survival: the Surveillance, Epidemiology, and End Results Program at the National Cancer Institute. J Natl Cancer Inst 97: 966-975

Harris JR, Lippman ME, Veronesi U, Willett W (1992): Breast cancer (1). *N Engl J Med* 327: 319–328

Hemminki K, Czene K (2002): Attributable risks of familial cancer from the Family-Cancer Database. Cancer Epidemiol Biomarkers Prev 11: 1638–1644

Hemminki K, Dong C (1999): Detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 91: 21 – 26.

Henderson BE, Bernstein L (1991): The international variation in breast cancer rates: an epidemiological assessment. Br Cancer Res Treat 18: S11–S17

Henninghausen L, Robinson GW (1998): Think globally, act locally: the making of a mouse mammary gland. Gnes Dev 12: 449–455.

Herrmann K, Niedobitek G (2003): Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. Breast Cancer Res 5: 13-17.

Hiatt R, Krieger N, Lobaugh B, Drezner M, Vogelman J, Orentreich N (1998): Prediagnostic serum vitamin D and breast cancer. *J Natl Cancer Inst* 90: 461–463.

Hilakivi-Clarke L, Luoto R, Huttunen T, Koskenvuo M (2005): Pregnancy weight gain and premenopausal breast cancer risk. J Reprod Med 50: 811-816.

Holick MF (2003): Vitamin D: A millenium perspective.   J Cell Biochem 88: 296-307

Holick MF (2004): Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr 80: 1678–1688.

Holick MF (2007): Vitamin D deficiency. N Engl J Med 357; 266-281.

Holl K, Lundin E, Kaasila M, Grankvist K, Afanasyeva Y, Hallmans G, Lehtinen M, Pukkala E, Surcel HM, Toniolo P, Zeleniuch-Jacquotte A, Koskela P, Lukanova A (2008): Effect of long-term storage on hormone measurements in samples from pregnant women: the experience of the Finnish Maternity Cohort. Acta Oncol 47**:** 406-412.

Hollis BW (2000): Comparison of commercially available (125)I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. Clin Chem 46: 1657-1661.

Hollstein M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R, Harris CC (1996): Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res 24: 141–146.

Holmøy T (2008): Vitamin D status modulates the immune response to Epstein Barr virus: Synergistic effect of risk factors in multiple sclerosis. Med Hypotheses 70: 66 – 69.

IARC (2008): Vitamin D and Cancer. *IARC Working Group Reports.* International Agency for Research on Cancer. *vol 5*. Lyon, France

Ibrahim EM, Ezzat AA, Baloush A, Hussain ZH, Mohammed GH (2000): Pregnancy-associated breast cancer: a case-control study in a young population with a high-fertility rate. Med Oncol 17: 293-300.

Inman GJ, Binné UK, Parker GA, Farrell PJ, Allday MJ (2001): Activators of the Epstein-Barr virus lytic program concomitantly induce apoptosis, but lytic gene expression protects from cell death. J Virol 75: 2400-2410

International Agency for Research on Cancer: *Weight Control and Physical Activity. Lyon, France: IARC Press* 2002.

Isabel Dos Santos Siva, 1999, pp. 182-183.

Ishida T, Yokoe T, Kasumi F, Sakamoto G, Makita M, Tominaga T, Simozuma K, Enomoto K, Fujiwara K, Nanasawa T, et al. Clinicopathologic characteristics and prognosis of breast cancer patients associated with pregnancy and lactation (1992): analysis of case-control study in Japan. Jpn J Cancer Res. 1992; 83: 1143-1149.

Izumi KM (2010): 'The EBV Latent Membrane Protein 1 oncoprotein' in Epstein-Barr virus: Latency and Transformation. Robertson ES, Caister Acad Press. pp 117

Jansen-van der Weide MC, Greuter MJ, Jansen L, Oosterwijk JC, Pijnappel RM, de Bock GH (2010): Exposure to low-dose radiation and the risk of breast cancer among women with a familial or genetic predisposition: a meta-analysis. Eur Radiol 20: 2547 - 2556.

Johannsson O, Loman N, Borg A, Olsson H (1998): Pregnancy-associated breast cancer in BRCA1 and BRCA2 germline mutation carriers. Lancet 352: 1359-1360

Johansson ENB (1979): Plasma levels of progesterone in pregnancy measured by a rapid competitive protein binding technique. Acta Endocrinol *61*: *607*-617

John E, Schwartz G, Dreon D, Koo J (1999): Vitamin D and breast cancer risk: the NHANES I epidemiologic follow-up study, 1971–1975 to 1992. Cancer Epidemiol Biomarkers Prev 8: 399–406

Joshi D, Quadri M, Gangane N, Joshi R, Gangane N (2009): Association of Epstein Barr virus infection (EBV) with breast cancer in rural Indian women. *PLoS One* 4: e8180, doi: 10.1371/journal.pone.0008180

Kaaks R, Berrino F, Key T, Rinaldi S, Dossus L, Biessy C, Secreto G, Amiano P, Bingham S, Boeing H, Bueno de Mesquita HB, Chang-Claude J, Clavel-Chapelon F, Fournier A, van Gils CH, Gonzalez CA, Gurrea AB, Critselis E, Khaw KT, Krogh V, Lahmann PH, Nagel G, Olsen A, Onland-Moret NC, Overvad K, Palli D, Panico S, Peeters P, Quirós JR, Roddam A, Thiebaut A, Tjønneland A, Chirlaque MD, Trichopoulou A, Trichopoulos D, Tumino R, Vineis P, Norat T, Ferrari P, Slimani N, Riboli E (2005): Serum sex steroids in premenopausal women and breast cancer risk within the European

Prospective Investigation into Cancer and Nutrition (EPIC). J Natl Cancer Inst 97: 755-765.

Karjalainen S (1990): Geographical variation in cancer patient survival in Finland: chance, confounding, or effect of treatment? J Epidemiol Community Health 44: 210-214.

Kazemi A, Sharifi F, Jafari N, Mousavinasab N (2009): High prevalence of vitamin D deficiency among pregnant women and their newborns in an Iranian population. J Womens Health 18: 835 –839

Keating NL, Cleary PD, Rossi AS, Zaslavsky AM, Ayanian JZ (1999): Use of hormone replacement therapy by postmenopausal women in the United States. Ann Intern Med 130: 545–553

Key J, Hodgson S, Omar RZ, Jensen TK, Thompson SG, Boobis AR, Davies DS, Elliott P (2006): Meta-analysis of studies of alcohol and breast cancer with consideration of the methodological issues. Cancer Causes Control 17: 759-770.

Key TJ (1995): Hormones and cancer in humans. Mutat Res 333: 59–67

Kieff E, Johannsen E, Calderwood MA (2010): Latent Epstein - Barr virus Infections . In Epstein - Barr virus Latency and Transformation by Robertson ES, Caister Academic Press: pp 3.

Koprowski C, Ross R, Mack W, Henderson B, Bernstein L (1999). Diet, body size and menarche in a multiethnic cohort. Br J Cancer 79: 1907-1911.

Koskela P, Anttila T, Bjørge T, Brunsvig A, Dillner J, Hakama M, Hakulinen T, Jellum E, Lehtinen M, Lenner P, Luostarinen T, Pukkala E, Saikku P, Thoresen S, Youngman L, Paavonen J (2000): Chlamydia trachomatis infection as a risk factor for invasive cervical cancer. Int J Cancer 85: 35 – 39.

Kroman N & Mouridsen HT (2003): Prognostic influence of pregnancy before, around, and after diagnosis of breast cancer. Breast 12: 516–521.

Labrecque LG, Barnes DM, Fentiman IS, Griffin BE (1995): Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Res 55: 39 – 45

Labrecque S, Naor N, Thomson D, Matlashewski G (1993): Analysis of the anti-p53 response in cancer patients. Cancer Res 53: 3468-3471

Lagiou A, Lagiou P, Vassilarou DS, Stoikidou M, Trichopoulos D (2003): Comparison of age at first full-term pregnancy between women with breast cancer and women with benign breast diseases. Int J Cancer 107: 817-821.

Lajous M and Zhang SM (2010): Diet and Nutrition in Molecular Epidemiology: Principles and practices. pp 173-174.

Lambe M, Ekbom A (1995): Cancers coinciding with childbearing: delayed diagnosis during pregnancy? BMJ 311: 1607-1608.

Lambe M, Hsieh C, Trichopoulos D, Ekbom A, Pavia M, Adami HO (1994): Transient increase in the risk of breast cancer after giving birth. N Engl J Med 331: 5-9.

Landi MT, Caporaso N (1997): Sample collection, processing and storage. IARC Sci Publ 142 :223-36

Langseth H, Luostarinen T, Bray F, Dillner J (2010): Ensuring quality in studies linking cancer registries and biobanks. Acta Oncol 49: 368-377

Larsson SC, Akesson A, Bergkvist L, Wolk A (2010): Multivitamin use and breast cancer incidence in a prospective cohort of Swedish women. Am J Clin Nutr 91:1268-1272.

Lehtinen M, Koskela P, Ogmundsdottir HM, Bloigu A, Dillner J, Gudnadottir M, Hakulinen T, Kjartansdottir A, Kvarnung M, Pukkala E, Tulinius H, Lehtinen T (2003): Maternal herpesvirus infections and risk of acute lymphoblastic leukemia in the offspring. Am J Epidemiol 158: 207 – 13.

Lemire J (2000): 1, 25-Dihydroxyvitamin D3--a hormone with immunomodulatory properties. Z Rheumatol 59: 24 –7.

Lenner P, Wiklund F, Emdin SO, Arnerlöv C, Eklund C, Hallmans G, Zentgraf H, Dillner J (1999). Serum antibodies against p53 in relation to cancer risk and prognosis in breast cancer: a population-based epidemiological study. Br J Cancer 79: 927- 932.

Levine AJ (1997): p53, the cellular gatekeeper for growth and division. Cell 88: 323–331.

Levine M, Moutquin JM, Walton R, Feightner J (2001): Chemoprevention of breast cancer. CMAJ 164: 1681-90

Lin Z, Flemington EK (2010): Regulation of EBV latency by viral lytic proteins. In Epstein - Barr virus Latency and Transformation by Robertson ES, Caister Academic Press: pp 178.

Liu Q, Wuu J, Lambe M, Hsieh SF, Ekbom A, Hsieh CC (2002): Transient increase in breast cancer risk after giving birth: postpartum period with the highest risk (Sweden). Cancer Causes Control 13: 299–305.

Lou YR, Laaksi I, Syvälä H, Bläuer M, Tammela TL, Ylikomi T, Tuohimaa P (2004): 25-hydroxyvitamin D3 is an active hormone in human primary prostatic stromal cells. FASEB J 18: 332-334.

Lowe L, Hansen CM, Senaratne S, Colston KW (2003): Mechanisms implicated in the growth regulatory effects of vitamin D compounds in breast cancer cells. Recent Results Cancer Res 164: 99–110.

Lukanova A, Andersson R, Wulff M, Zeleniuch-Jacquotte A, Grankvist K, Dossus L, Afanasyeva Y, Johansson R, Arslan AA, Lenner P, Wadell G, Hallmans G, Toniolo P, Lundin E (2008): Human chorionic gonadotropin and alpha-fetoprotein concentrations in pregnancy and maternal risk of breast cancer: a nested case-control study. Am J Epidemiol 168: 1284-1291.

Ma H, Henderson KD, Sullivan-Halley J, Duan L, Marshall SF, Ursin G, Horn-Ross PL, Largent J, Deapen DM, Lacey JV Jr, Bernstein L (2010): Pregnancy-related factors and the risk of breast carcinoma in situ and invasive breast cancer among postmenopausal women in the California Teachers Study cohort. Breast Cancer Res 12: R35

Männistö T, Surcel HM, Bloigu A, Ruokonen A, Hartikainen AL, Järvelin MR, Pouta A, Vääräsmäki M, Suvanto-Luukkonen E (2007): The effect of freezing, thawing, and short- and long-term storage on serum thyrotropin, thyroid hormones, and thyroid autoantibodies: implications for analyzing samples stored in serum banks. Clin Chem 53: 1986-1987.

Maskarinec G, Morimoto Y, Takata Y, Murphy SP, Stanczyk FZ (2006): Alcohol and dietary fibre intakes affect circulating sex hormones among premenopausal women. Public Health Nutr 9: 875-881.

McCullough ML, Stevens VL, Patel R, Jacobs EJ, Bain EB, Horst RL, Gapstur SM, Thun MJ, Calle EE (2009): Serum 25-hydroxyvitamin D concentrations and postmenopausal breast cancer risk: a nested case control study in the Cancer Prevention Study-II Nutrition Cohort. Breast Cancer Res 11: R64

McMurray HR, Sampson ER, Compitello G, Kinsey C, Newman L, Smith B, Chen SR, Klebanov L, Salzman P, Yakovlev A, Land H (2008): Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. Nature 453: 1112-1116.

Merkel DE (1996). Pregnancy and breast cancer. Semin Surg Oncol 12: 370- 375.

Metcalfe S, Wheeler TK, Picken S, Negus S, Jo Milner A (2000). P53 autoantibodies in 1006 patients followed up for breast cancer. Breast Cancer Res 2: 438-443.

Meyer F, Moisan J, Marcoux D, Bouchard C (1990): Dietary and physical determinants of menarche. Epidemiology 1:377-381.

Middleton LP, Amin M, Gwyn K, Theriault R, Sahin A (2003): Breast carcinoma in pregnant women: assessment of clinicopathologic and immunohistochemical features. Cancer 98:1055-1060.

Miller WR (1993): Hormonal factors and risk of breast cancer. Lancet 341: 25-26.

Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR (1993): Interleukin-10. Annu Rev Immunol 11:165- 190.

Mori S, Ito G, Usami N, Yoshioka H, Ueda Y, Kodama Y, Takahashi M, Fong KM, Shimokata K, Sekido Y (2004): p53 apoptotic pathway molecules are frequently and simultaneously altered in non-small cell lung carcinoma. Cancer 100: 1673-1682.

Murray PG (2006): Epstein-Barr virus in breast cancer: artefact or aetiological agent? J Pathol 209: 427 – 429.

Murray PG, Lissauer D, Junying J, Davies G, Moore S, Bell A, Timms J, Rowlands D, McConkey C, Reynolds GM, Ghataura S, England D, Caroll R, Young LS (2003): Reactivity with a monoclonal antibody to Epstein–Barr virus nuclear antigen 1 defies a subset of aggressive breast cancers in the absence of the EBV genome. Cancer Res 63: 2338–2343.

Nakamura N, Miyazaki K, Kitano Y, Fujisaki S, Okamura H (1993): Suppression of cytotoxic T-lymphocyte activity during human pregnancy. J Reprod Immunol 23: 119-130.

National breast and ovarian cancer center (2009): Breast cancer risk factors: A review of the evidence. www.nbocc.org.au.

Nelson WG, Kastan MB (1994): DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 14: 1815–1823.

Newman B, Mu H, Butler LM, Millikan RC, Moorman PG, King MC (1998): Frequency of breast cancer attributable to BRCA1 in a population-based series of American women. JAMA 279: 915–921.

NIH, USA (1999): Stages of Breast development: normal to metastatic disease.

NORDCAN- Associationof Nordic Cancer Registries (2010). http://www-dep.iarc.fr/nordcan.htm

Onitilo AA, Engel JM, Greenle RT, Mukesh BN (2009): Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clin Med Res 7: 4-13.

Opatrny L, Dell'Aniello S, Assouline S, Suissa S (2008): Hormone replacement therapy use and variations in the risk of breast cancer. BJOG 115: 169-175

Osborne C, Wilson P, Tripathy D (2004): Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. Oncologist 9: 361-377.

Parise CA, Bauer KR, Brown MM, Caggiano V (2009): Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999-2004. Breast J 15: 593-602.

Parkin DM, Bray F, Ferlay, Pisani P (2005): Global cancer statistics , 2002. CA *Cancer J Clin* 55 : 74–108.

Parkin DM, Fernández LMG (2006): Use of statistics to assess the global burden of breast cancer. *Breast J* 12: S70–S80.

Pathak DR (2002): Dual effect of first full term pregnancy on breast cancer risk: empirical evidence and postulated underlying biology. Cancer Causes Control 13: 295-298.

Peng X, Vaishnav A, Murillo G, Alimirah F, Torres KE, Mehta RG (2010): Protection against cellular stress by 25-hydroxyvitamin D(3) in breast epithelial cells. J Cell Biochem 110: 1324-1333.

Perrigoue JG, den Boon JA, Friedl A, Newton MA, Ahlquist P, Sugden B (2005): Lack of association between EBV and breast carcinoma. Cancer Epidemiol Biomarkers Prev 14: 809-814.

Petridou E, Katsouyanni K, Spanos E, Skalkidis Y, Panagiotopoulou K, Trichopoulos D (1992): Pregnancy estrogens in relation to coffee and alcohol intake. Ann Epidemiol 1992; 2: 241-247

Pike MC, Krailo MD, Henderson BE, Casagrande JT, Hoel DG (1983): Hormonal risk factors, breast tissue age and the age-incidence of breast cancer. *Nature* 303:767-770.

Powles T, Eeles R, Ashley S, Easton D, Chang J, Dowsett M, Tidy A, Viggers J, Davey J (1998): Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomised chemoprevention trial. Lancet 352: 98-101.

Prentice A (2008): Vitamin D deficiency: a global perspective. Nutr Rev 66: S153-64.

Preston DL, Mattsson A, Holmberg E, Shore R, Hildreth NG, Boice JD Jr (2002): Radiation effects on breast cancer risk: a pooled analysis of eight cohorts. Radiat Res 158: 220–235.

Psyrri A, Burtness B (2005): Pregnancy-associated breast cancer. Cancer J 11: 83-95.

Pukkala E, Andersen A, Berglund G, Gislefoss R, Gudnason V, Hallmans G, Jellum E, Jousilahti P, Knekt P, Koskela P, Kyyrönen PP, Lenner P, Luostarinen T, Löve A, Ogmundsdóttir H, Stattin P, Tenkanen L, Tryggvadóttir L, Virtamo J, Wadell G, Widell A, Lehtinen M, Dillner J (2007): Nordic biological specimen banks as basis for studies of cancer causes and control--more than 2 million sample donors, 25 million person years and 100,000 prospective cancers. Acta Oncol 46: 286-307.

Rajkumar L, Kittrell FS, Guzman RC, Brown PH, Nandi S, Medina D (2007): Hormone-induced protection of mammary tumorigenesis in genetically engineered mouse models. Breast Cancer Res 9 :R12.

Richardson AK, Cox B, McCredie MR, Dite GS, Chang JH, Gertig DM, Southey MC, Giles GG, Hopper JL (2004): Cytomegalovirus, Epstein-Barr virus and risk of breast cancer before age 40 years: a case-control study. Br J Cancer 90: 2149 –52.

Rohan TE, Hartwick W, Miller AB, Kandel RA (1998): Immunohistochemical detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. J Natl Cancer Inst 90:1262–1269.

Rohan TE, Jain M, Howe GR, Miller AB (2000): Alcohol consumption and risk of breast cancer: a cohort study.Cancer Causes Control 11: 239- 247.

Rohan TE, Negassa A, Caan B, Chlebowski RT, Curb JD, Ginsberg M, Lane DS, Neuhouser ML, Shikany JM, Wassertheil-Smoller S, Page DL (2008): Low-fat dietary pattern and risk of benign proliferative breast disease: a randomized, controlled dietary modification trial. Cancer Prev Res (Phila Pa) 1:275- 278.

Rohan TE, Negassa A, Chlebowski RT, Ceria-Ulep CD, Cochrane BB, Lane DS, Ginsberg M, Wassertheil-Smoller S, Page DL (2009): A randomized controlled trial of calcium plus vitamin D supplementation and risk of benign proliferative breast disease. Breast Cancer Res Treat 116 : 339-350.

Ronckers CM, Doody MM, Lonstein JE, Stovall M, Land CE (2008): Multiple diagnostic X-rays for spine deformities and risk of breast cancer. Cancer Epidemiol Biomarkers Prev. 2008; 17: 605–613.

Ronckers CM, Erdmann CA, Land CE(2005): *Radiation and breast cancer: a review of current evidence.* Breast Cancer Res *7:21–32.*

Ross RK, Paganini-Hill A, Wan PC, Pike MC (2000): Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J Natl Cancer Inst 92: 328- 332.

Russo IH, Koszalka M, Gimotty PA, Russo J (1990-A): Protective effect of chorionic gonadotropin on DMBA-induced mammary carcinogenesis. Br J Cancer 62: 243-247.

Russo IH, Koszalka M, Russo J (1990-B). Effect of human chorionic gonadotropin on mammary gland differentiation and carcinogenesis. Carcinogenesis 11: 1849-1855.

Russo IH, Russo J (1996): Mammary gland neoplasia in long-term rodent studies. Environ Health Perspect 104: 938-967.

Russo J, Balogh GA, Russo IH (2008): Full-term pregnancy induces a specific genomic signature in the human breast. Cancer Epidemiol Biomarkers Pre 17: 51-66

Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ (1990) : Comparative study of human and rat mammary tumorigenesis. Lab Invest Mar 62: 244–278.

Russo J, Lynch H, Russo IH (2001): Mammary gland architecture as a determining factor in the susceptibility of the human breast to cancer. Breast J 7: 278- 291.

Russo J, Russo IH, Rogers AE, van Zwieten MJ, Gusterson B (1990): Pathology of tumours in laboratory animals. Tumours of the rat. Tumours of the mammary gland. IARC Sci Publ 99:47–78.

Russo J, Tay LK, Russo IH (1982): Differentiation of the mammary gland and susceptibility to carcinogenesis. *Breast Cancer Res. Treat 2*: 5-73 (Review).

Schedin P (2006): Pregnancy-associated breast cancer and metastasis. Nat Rev Cancer 6: 281– 291 (Review).

Schneider HP, Mueck AO, Kuhl H (2005): IARC monographs program on carcinogenicity of combined hormonal contraceptives and menopausal therapy. Climacteric 8: 311-316.

Shin HR, Joubert C, Boniol M, Hery C, Ahn SH, Won YJ, Nishino Y, Sobue T, Chen CJ, You SL, Mirasol-Lumague MR, Law SC, Mang O, Xiang YB, Chia KS, Rattanamongkolgul S, Chen JG, Curado MP, Autier P (2010): Recent trends and patterns in breast cancer incidence among Eastern and Southeastern Asian women. Cancer Causes Control 21: 1777-1785.

Siegelmann-Danieli N, Tamir A, Zohar H, Papa MZ, Chetver LL, Gallimidi Z, Stein ME, Kuten A (2003): Breast cancer in women with recent exposure to fertility medications is associated with poor prognostic features. Ann Surg Oncol 10:1031-1038.

Silva IS (1999b): *Cancer epidemiology: principles and methods.* Lyon: IARC Scientific Publications pp 182-183.

Sivaraman L, Conneely OM, Medina D, O'Malley BW (2001): p53 is a potential mediator of pregnancy and hormone-induced resistance to mammary carcinogenesis. Proc Natl Acad Sci U S A 98: 12379- 12384.

Slattery ML, Kerber RA (1993): A comprehensive evaluation of family history and breast cancer risk. The Utah Population Database. JAMA 270 :1563-1568.

Smith RA (2002): IARC Working group on the Evaluation of Cancer Preventive Strategies Breast cancer screening. IARC Handbooks of Cancer Prevention, vol 7. IARC Press, Lyon.

Smith-Warner SA, Spiegelman D, Yaun SS, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Holmberg L, Howe GR, Marshall JR, Miller AB, Potter JD, Speizer FE, Willett WC, Wolk A, Hunter DJ (1998): Alcohol and breast cancer in women: a pooled analysis of cohort studies. JAMA 279(7): 535-540.

Steen HB (2000): The origin of oncogenic mutations: where is the primary damage? Carcinogenesis 21: 1773- 1776.

Steinberg KK, Thacker SB, Smith SJ, Stroup DF, Zack MM, Flanders WD, Berkelman RL (1991): A meta-analysis of the effect of estrogen replacement therapy on the risk of breast cancer. JAMA 265: 1985–1990.

Stevens SJ, Smits PH, Verkuijlen SA, Rockx DA, van Gorp EC, Mulder JW, Middeldorp JM (2007): Aberrant Epstein-Barr virus persistence in HIV carriers is characterized by anti-Epstein-Barr virus IgA and high cellular viral loads with restricted transcription.AIDS. 21: 2141-2149.

Stewart BW, Kleihues P (2003): World Cancer Report 2003 .IARC Publications.

Stolzenberg-Solomon RZ, Hayes RB, Horst RL, Anderson KE, Hollis BW, Silverman DT (2009): Serum vitamin D and risk of pancreatic cancer in the prostate, lung, colorectal, and ovarian screening trial. Cancer Res 69:1439- 1447.

Stolzenberg-Solomon RZ, Jacobs EJ, Arslan AA, Qi D, Patel AV, Helzlsouer KJ, Weinstein SJ, McCullough ML, Purdue MP, Shu XO, Snyder K, Virtamo J, Wilkins LR, Yu K,

Zeleniuch-Jacquotte A, Zheng W, Albanes D, Cai Q, Harvey C, Hayes R, Clipp S, Horst RL, Irish L, Koenig K, Le Marchand L, Kolonel LN (2010): Circulating 25-hydroxyvitamin D and risk of pancreatic cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. Am J Epidemiol 172: 81-93.

Stone J, Ding J, Warren RM, Duffy SW, Hopper JL (2010): Using mammographic density to predict breast cancer risk: dense area or percent dense area. Breast Cancer Res 12: R97

Teppo L, Pukkala E, Lehtonen M (1994). Data quality and quality control of a population-based cancer registry. Experience in Finland. Acta Oncol 33: 365-69.

Terry MB, Zhang FF, Kabat G, Britton JA, Teitelbaum SL, Neugut AI, Gammon MD (2006): Lifetime alcohol intake and breast cancer risk. Ann Epidemiol 16 :230-40.

The Lancet (2009): Breast cancer in developing countries 374: 9701. DIO:10.1016/S0140-6736(09)61930-9

Thomas HV, Key TJ, Allen DS, Moore JW, Dowsett M, Fentiman IS, Wang DY (1997): A prospective study of endogenous serum hormone concentrations and breast cancer risk in premenopausal women on the island of Guernsey. Br J Cancer 75: 1075–9

Toniolo P, Grankvist K, Wulff M, Chen T, Johansson R, Schock H, Lenner P, Hallmans G, Lehtinen M, Kaaks R, Wadell G, Zeleniuch-Jacquotte A, Lundin E, Lukanova A (2010): Human Chorionic Gonadotropin in Pregnancy and Maternal Risk of Breast Cancer. Cancer Res 70: 6779-6786.

Travis LB, Hill D, Dores GM, Gospodarowicz M, van Leeuwen FE, Holowaty E, Glimelius B, Andersson M, Pukkala E, Lynch CF, Pee D, Smith SA, Van't Veer MB, Joensuu T, Storm H, Stovall M, Boice JD Jr, Gilbert E, Gail MH (2005): Cumulative absolute breast cancer risk for young women treated for Hodgkin lymphoma. *J Natl Cancer Inst* 97: 1428–1437.

Tsoukas CD, Provvedini DM, Manolagas SC (1984): 1,25-dihydroxyvitamin D3: a novel immunoregulatory hormone. Science 224:1438-1440.

Tuohimaa P, Tenkanen L, Ahonen M, Lumme S, Jellum E, Hallmans G, Stattin P, Harvei S, Hakulinen T, Luostarinen T, Dillner J, Lehtinen M, Hakama M (2004): Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a

longitudinal, nested case-control study in the Nordic countries. Int J Cancer 108**:** 104-108.

Ursin G, Hovanessian-Larsen L, Parisky YR, Pike MC, Wu AH (2005): Greatly increased occurrence of breast cancers in areas of mammographically dense tissue. Br Cancer Res 7: 605–608.

Vainio H, Kaaks R and Bianchini F (2002): Weight control and physical activity in cancer prevention: international evaluation of the evidence. Eur J Cancer Prev 11: 94-00.

Vehmanen P, Friedman LS, Eerola H, McClure M, Ward B, Sarantaus L, Kainu T, Syrjäkoski K, Pyrhönen S, Kallioniemi OP, Muhonen T, Luce M, Frank TS, Nevanlinna H (1997): Low proportion of BRCA1 and BRCA2 mutations in Finnish breast cancer families: evidence for additional susceptibility genes. Hum Mol Genet 6 :2309- 2315.

Veronesi U, Maisonneuve P, Costa A, Sacchini V, Maltoni C, Robertson C, Rotmensz N, Boyle P (1998): Prevention of breast cancer with tamoxifen: preliminary findings from the Italian randomised trial among hysterectomised women. Italian Tamoxifen Prevention Study.Lancet 352: 93-97.

Visvanathan K, Chlebowski RT, Hurley P, Col NF, Ropka M, Collyar D, Morrow M, Runowicz C, Pritchard KI, Hagerty K, Arun B, Garber J, Vogel VG, Wade JL, Brown P, Cuzick J, Kramer BS, Lippman SM (2009): American society of clinical oncology clinical practice guideline update on the use of pharmacologic interventions including tamoxifen, raloxifene, and aromatase inhibition for breast cancer risk reduction. J Clin Oncol 27: 3235- 3258.

Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH (2002): An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development 129:1377-1386.

Wakai K, Dillon DS, Ohno Y, Prihartono J, Budiningsih S, Ramli M, Darwis I, Tjindarbumi D, Tjahjadi G, Soetrisno E, Roostini ES, Sakamoto G, Herman S, Cornain S (2000): Fat intake and breast cancer risk in an area where fat intake is low: a case–control study in Indonesia. Int J Epidemiol 29: 20–28.

Webb AR (2006): Who, what, where and when—influences on cutaneous vitamin D synthesis. Prog Biophys Mol Biol 92: 17–25.

Webb PM, Byrne C, Schnitt SJ, Connolly JL, Jacobs TW, Baer HJ, Willett WC, Colditz GA (2004): A prospective study of diet and benign breast disease. Cancer Epidemiol Biomarkers Prev 13: 1106–1113.

Welsh JE (2004): Vitamin D and breast cancer: insights from animal models. Am J Clin Nutr 80:1721- 1724.

Welsh J, Wietzke JA, Zinser GM, Byrne B, Smith K, Narvaez CJ (2003). Vitamin D-3 receptor as a target for breast cancer prevention. J Nutr 133: 2425 –2433.

Willett AM (2005): Vitamin D status and its relationship with parathyroid hormone and bone mineral status in older adolescents. Proc Nutr Soc 64:193–203.

Winn DM, Reichman ME, Gunter E (1990): Epidemiologic issues in the design and use of biologic specimen banks. Epidemiol Rev 12: 56-70.

Wohlfahrt J, Melbye M (2001): Age at any birth is associated with breast cancer risk. Epidemiology 12: 68-73.

Wong NS, Anderson BO, Khoo KS, Ang PT, Yip CH, Lu YS, Voravud N, Shao ZM, Pritchard KI; Asian Oncology Summit (2009): Management of HER2-positive breast cancer in Asia: consensus statement from the Asian Oncology Summit. Lancet Oncol 10: 1077-1085.

World Health Organ Tech Rep Ser (2003): Diet, nutrition and the prevention of chronic diseases 916:1-149.

Wu AH, Pike MC, Stram DO (1999): Meta-analysis: dietary fat intake, serum estrogen levels, and the risk of breast cancer. J Natl Cancer Inst: 91: 529-534.

Wu M, Mao C, Chen Q, Cu XW, Zhang WS (2010): Serum p53 protein and anti-p53 antibodies are associated with increased cancer risk: a case-control study of 569 patients and 879 healthy controls. Mol Biol Rep 37: 339-343.

Yin L, Grandi N, Raum E, Haug U, Arndt V, Brenner H (2010): Meta-analysis: Serum vitamin D and breast cancer risk. Eur J Cancer 46: 2196 – 2205.

Younes M, Lebovitz RM, Bommer KE, Cagle PT, Morton D, Khan S, Laucirica R (1995): p53 accumulation in benign breast biopsy specimens. Hum Pathol 26:155-158.

Yu CK, Sykes L, Sethi M, Teoh TG, Robinson S (2009): Vitamin D deficiency and supplementation during pregnancy. Clin Endocrinol 70:685 – 689.

Zhang S, Hunter DJ, Forman MR, Rosner BA, Speizer FE, Colditz GA, Manson JE, Hankinson SE, Willett WC (1999): Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. J Natl Cancer Inst 91: 547–556.

Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW, Wu-Williams AH, Kolonel LN, Horn-Ross PL, Rosenthal JF, Hyer MB (1993): Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 85: 1819-1827.

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The Effects of Storage Time and Sampling Season on the Stability of Serum 25-Hydroxy Vitamin D and Androstenedione

Calypse Agborsangaya*
University of Tampere, Tampere, Finland

Adetunji T. Toriola*
National Institute for Health and Welfare, Oulu, Finland

Kjell Grankvist
Umeå University, Umeå, Sweden

Heljia-Marja Surcel
National Institute for Health and Welfare, Oulu, Finland

Katsiaryna Holl, Seppo Parkkila, and Pentti Tuohimaa
University of Tampere, Tampere, Finland

Annekatrin Lukanova
German Cancer Research Centre, Heidelberg, Germany

Matti Lehtinen
University of Tampere, Tampere, Finland and National Institute for Health and Welfare, Oulu, Finland

    Knowledge of the stability of serum samples stored in large biobanks is pivotal for reliable assessment of hormone-dependent disease risks. We studied the effects of sample storage time and season of serum sampling on the stability of 25-hydroxy vitamin D (25-OHD) and androstenedione in a stratified random sample of 402 women, using paired sera from the Finnish Maternity Cohort. Serum samples selected were donated between 6 and 24 yr ago. The storage time did not affect serum 25-OHD and androstenedione levels. However, there was a significant mean difference in the 25-OHD levels of sera withdrawn during winter (first sample) vs. during summer (second sample; –18.4 nmol/l, $P \le 0.001$). Also at the individual level, there were significant differences in average 25-OHD levels between individuals with the paired sera taken at winter–winter compared with other alternatives (summer–winter, winter–summer, and summer–summer). The androstenedione levels showed no such differences. Long-term storage does not affect serum 25-OHD and androstenedione levels, but sampling season is an important determinant of 25-OHD levels. Stored serum samples can be used to study disease associations with both hormones. However, sampling season needs to be taken into account for 25-OHD by considering matching and stratification and, if possible, serial sampling.

INTRODUCTION

    Vitamin D is a "steroid-like" hormone, which is produced photochemically from 7-dehydrocholesterol on exposure of the skin to ultraviolet (UV) light. It can also be obtained from foods such as cod liver oil, salmon, eggs, liver, and so forth. Pre-vitamin D exists in 2 forms, ergocalciferol (vitamin D_2, from plant sources) and cholecalciferol (vitamin D_3, from animal sources). Once absorbed, vitamin D_3 undergoes hydroxylation in the liver to form 25-hydroxyvitamin (OH) D_3 (25-OHD_3, which is further hydroxylated to 1,25-dihydroxyvitamin D_3 (1,25 OH_2D_3) in the kidney, breast, colon, and prostate (1,2). The traditional function of 25-OHD is to maintain skeletal integrity by regulating calcium and phosphorus homeostasis (3,4). Epidemiological studies have associated low serum 25-OHD levels with a wide variety of diseases such as diabetes, some cancers, asthma, multiple sclerosis, rheumatoid arthritis, cardiovascular diseases, and schizophrenia (5).

Because of the wide range of genomic and nongenomic effects of 25-OHD and its metabolites, a number of studies are being conducted exploring the 25-OHD disease associations. Most of these studies have exploited serum samples that have been stored in biobanks for several years, even decades. Stored serum 25-OHD is unaffected by multiple freeze-thaw cycles (6) and is stable when stored at $-20°C$ for at least 3 yr (7). The impact of longer storage time has not been thoroughly considered. However, a recent study reported that maternal sera frozen for 40 yr could be used to detect seasonal and racial differences in 25-OHD concentrations (8). The serum 25-OHD concentrations in the 40-yr-old sera were, however, lower than those of 2-yr-old sera.

Androstenedione is a steroid hormone produced from the adrenals and ovaries. It is primarily synthesized from dehydroepiandrosterone (DHEA) and later reversibly converted to testosterone (9). Previous studies to determine seasonal changes in androgen levels have been equivocal (10–13).

The action of 25-OHD is androgen dependent in some organs such as the prostrate (14,15). An experimental study revealed that in intact animals, prostatic growth was significantly inhibited by $25-OHD_3$, but in castrated animals who have low androgens, 25-OHD had no effect on prostatic growth (16). At physiologic concentrations, $25-OHD_3$ exhibits strong antiproliferative activity only in the presence of dihydrotestosterone (14).

Using paired serum samples that have been stored for periods ranging from between 6 to 24 yr at the Finnish Maternity Cohort (FMC), we sought to determine the effects of storage time and sampling season on serum 25-OHD and androstenedione concentrations.

MATERIAL AND METHODS

FMC

The FMC contains 1.3 million first trimester serum samples from approximately 98% of all pregnant Finnish women. The samples have been collected since 1983. The samples are collected at municipal maternity care units following an informed consent during the first trimester of pregnancy for screening of congenital infections. After the screening tests have been done, the remaining sample (1–3 ml volume of serum) is stored at $-25°C$ in polypropylene cryo vials at the National Institute for Health and Welfare (formerly National Public Health Institute) in Oulu, Finland (17). Detailed demographic and reproductive history data are collected for each participant.

Sample Identification

The samples used for this study were randomly selected following the sampling applied in a previous study within the FMC (18). In that study, 10 random first pregnancy samples were retrieved for every other year from 1984 to 2002 (altogether 100 samples). For this study, we selected 4 comparable first preg-

nancy samples taking into account also the sampling season: two samples for the same season (summer) and two for the opposite season (winter), age ($+/-$ 4 yr), and postal code (municipality). We defined winter as the 15th of December to the 15th of March and summer as the 1st of May to the 31st of August. A total of 453 eligible individuals were identified from the FMC database. Of the corresponding 453 samples, eventually 402 samples were selected to make 201 summer samples and 201 winter samples appropriately matched for postal code and age. The lowest numbers of samples were from 1988 (37), and the highest numbers were from 1990, 1992, and 1996, with 42 samples each. From the final list of the 402 individuals, 100 individuals who had a second pregnancy within 5 yr of the first pregnancy were identified. We randomly selected 40 such individuals whose first and second pregnancies were from the same season (summer/summer and winter/winter) and 40 such individuals whose first and second pregnancy samples were from different seasons (summer/winter and winter/summer). Complete paired sample sets were available for 78 individuals.

Sample Handling

The available, previously unthawed samples were aliquoted into two 160 μl aliquots for 25-OHD and androstenedione analysis. Samples with indication of hemolysis or visual signs of freeze drying were not eligible. One aliquot per sample was sent to the Department of Anatomy, University of Tampere, Tampere, Finland, for 25-OHD analysis and another aliquot to the Clinical Chemistry Laboratory, Department of Medical Biosciences, Umeå University, Umeå, Sweden, for androstenedione analysis.

Laboratory Methods

Quantification of 25-OHD was done using an 25-OHD IDS-radioimmunoassay from IDS Ltd (Boldon, UK. The manufacturer stated a specificity (% cross-reactivity) of 100% for $25-OHD_3$; 75% for $25-OHD_2$; 100% for $24, 25-OH_2D_3$; and less than 0.01% or 0.3% for cholecalciferol (D_3) and ergocalciferol (D_2), respectively. The mean interassay CV was 4.0% for the first pregnancy samples and 3.4% for the second pregnancy samples at 25-OHD mean of 26.5 nmol/l and 103 nmol/l, respectively.

Determination of serum levels of androstenedione were performed at the Department of Medical Biosciences, University of Umeå, Umeå, Sweden. The steroid levels were measured by competitive chemiluminescent enzyme immunoassay (DPC Immulite 2000 Androstenedione Chemiluminescent EIA, UK). With control sera, the interassay CV was 16.9% at 2.2 ng/ml and 6.2% at 5.4 ng/ml.

The study was approved by the ethical committee of the National Institute for Health and Welfare, Finland.

Statistical Methods

We used SPSS (SPSS, Inc., Chicago, IL) version 14 for all statistical analysis. Serum 25-OHD was not normally

TABLE 1

Baseline characteristics of a stratified, random sample of women stemming from the Finnish Maternity Cohort with single and paired pregnancy samples

	1st Pregnancy Samples		1st and 2nd Pregnancy Samples	
Variable	Winter	Summer	Winter	Summer
Number (N)	163	161	38	40
Mean age (yr)	28.6	28.7	30.8	31.2
Mean storage time (yr)	14.9	14.9	13.2	12.3

distributed, and we performed a logarithmic transformation. Serum androstenedione was normally distributed. Clustered box plots were drawn for both and the duration of sample storage, stratified by sampling season. Pearson's partial correlation coefficient (r) was used to test for correlation between duration of sample storage and both 25-OHD and androstenedione. Independent sample t-test was used to assess the effect of sampling season on single hormone measurement, whereas paired sample t-test was used to assess the effect of the sampling season on paired hormone measurements.

RESULTS

The baseline characteristics (mean age, sample storage time) of the women who donated one or two pregnancy serum samples in different seasons were similar. For the first pregnancy samples, the mean and median ages at sample withdrawal were 28.7 and 29 yr, respectively (range = 22–38 yr). Mean duration of storage was 14.9 yr, ranging from 6 yr to 24 yr. For the second pregnancy samples, the mean and median ages were 31.0 and 30.4 yr, respectively (range = 25–40 yr). Their mean duration of storage was 12.8 yr, ranging from 4 to 22 yr (Table 1).
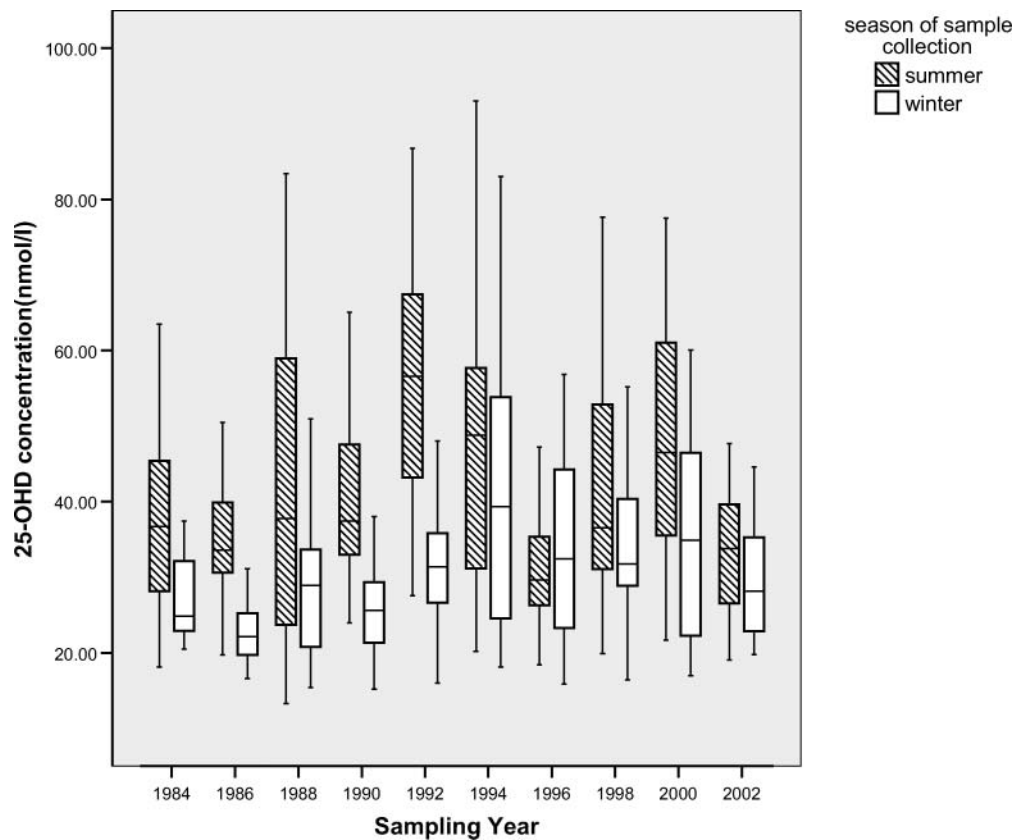

FIG. 1. Serum 25-hydroxy vitamin D (25-OHD; nmol/l) in a stratified, random sample of pregnant women ($n = 402$) stemming from the Finnish Maternity Cohort (1984–2002) by sampling season. The median serum 25-OHD levels are significantly higher in summer than in winter. The error bars represent the 25th (lower) and 75th (upper) percentiles.
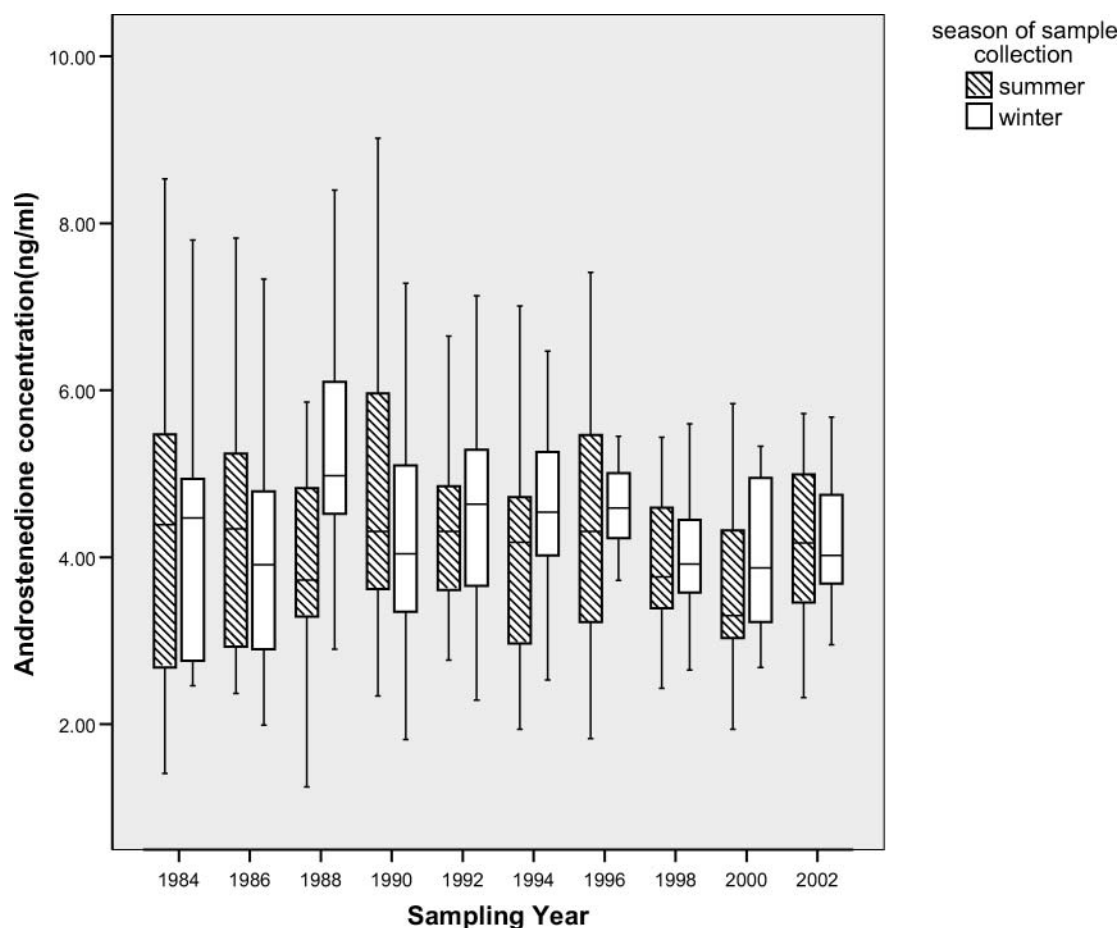
FIG. 2. Serum androstenedione (ng/ml) in a stratified, random sample of pregnant women stemming from the Finnish Maternity Cohort (1984–2002) by sampling season. The median serum androstenedione levels appear to be higher in winter than in summer. The error bars represent the 25th (lower) and 75th (upper) percentiles.

The highest average levels of serum 25-OHD were found in 1992 [44.8 nmol/l, 95% CI = 39.2–50.5) and 1994 (44.5 nmol/l, 95% CI = 38.8–50.2) and the lowest in 1996 (34.2 nmol/l, 95% CI = 30.4–38.0) and 2002 samples (34.7 nmol/l, 95% CI = 30.1–39.3). For the first pregnancy samples, mean serum 25-OHD levels were significantly lower in winter than in summer; 33.4 nmol/l (95% CI = 31.5–35.3) vs. 44.0 nmol/l (95% CI = 41.4–46.5; $P \leq 0.001$), respectively. This was true also for the second pregnancy samples; 39.8 nmol/l (95% CI = 46.7–56.5) vs. 51.6 nmol/l (95% CI = 34.5–45.0; $P \leq 0.001$), respectively. When stratified by the sampling season, the average annual serum 25-OHD levels were lower in the winter than in the summer (Figs. 1 and 2). The mean androstenedione levels appeared to be higher in winter than in summer (mean difference $\mu_d = 0.3$ nmol/ml, 95% CI = 0.0–0.6; P value = 0.05).

Serum 25-OHD levels showed practically no correlation with storage time for the first pregnancy samples ($r_s = -0.08$, P value = 0.1) or for the second pregnancy samples ($r_s = -0.09$, P value = 0.5). Furthermore, there was no correlation between the storage time and serum 25-OHD levels after controlling for

sampling age in either the first ($r_s = -0.02$, P value = 0.7) or in the second pregnancy samples ($r_s = -0.08$, P value = 0.51). Age adjusted androstenedione levels had no significant correlation with storage time ($r_s = 0.09$).

We found a highly significant mean difference in the 25-OHD levels of the paired sera withdrawn during winter (first sample) vs. during summer (second sample; mean difference $\mu_d = -18.4$ nmol/l; P value ≤ 0.001; Table 2). This was true (albeit to the opposite direction) also for individuals with the first sampling season at summer and the second sampling season at winter ($\mu_d = 9.4$ nmol/l; P value < 0.1; Table 2). Comparing the average serum 25-OHD levels for groups of individuals with different kinds of pairs of sampling seasons (identical or opposite seasons), we found significant differences when winter–winter samples were compared to all other types of sample pairs (Table 3). The highest differences were observed when comparing the average serum 25-OHD levels for winter–winter samples with summer–summer ($\mu_d = 16.6$ nmol/l, P value ≤ 0.001) and summer–winter samples ($\mu_d = 17.8$ nmol/l; P value ≤ 0.001). No significant differences were observed

TABLE 2

Mean differences in serum 25-OHD (nmol/l) in paired sera of pregnant Finnish women by season of sample withdrawal[a]

Seasons of Sample Withdrawal	Number (N)	Mean Differences Between 1st and 2nd Samples	95% Confidence Interval	P Value
Winter–Summer	19	−18.4	−25.05 to −11.68	≤ 0.001
Summer–Winter	15	9.4	−2.07 to 20.85	0.10
Summer–Summer	19	−1.9	−10.81 to 7.04	0.67
Winter–Winter	21	−5.1	−10.55 to 0.36	0.07

[a]Abbreviation is as follows: 25-OHD, 25-hydroxy vitamin D.

when similar comparisons were made for androstenedione levels (Table 3).

DISCUSSION

Long-term storage of serum samples in biobanks at –25°C had no effect on 25-OHD and androstenedione concentrations. Sampling season, however, had an effect. The average 25-OHD concentrations were significantly lower in single and paired samples taken during winter than in summer. If anything, androstenedione concentrations tended to show the opposite.

The results from our study confirm previous studies on vitamin D stability. Ocke et al. (7) analyzed serum vitamin D concentrations of the same individual at intervals during 4 yr and found approximately 10% difference (increase or decrease) in mean vitamin D concentrations at given time points. They concluded that the differences are most likely due to systematic differences in laboratory measurements. Bodnar et al. (8) compared 25-OHD concentrations in sera stored for 40 yr with those of sera stored for 2 yr in different individuals. Even though

the mean 25-OHD concentrations in the 40-yr-old samples were lower than those of the 6-yr-old samples, the measurements were similar in both. Seasonal and racial differences in mean 25-OHD concentrations were found between both cohorts, implying that if there was any deterioration in 25-OHD detectability, it was similar across all the samples (8).

The first pregnancy samples of our cohort had been drawn in paired years between 1984 and 2002 (6–24 yr ago). We did not observe any correlation between serum 25-OHD concentrations and the sampling time. However, we observed a consistent statistically significant association between 25-OHD concentrations and the sampling season. Also in the paired samples of the same individuals, mean serum 25-OHD concentrations were always higher in summer than in winter, an observation that has been previously well documented in single sample analyses (8,19,20).

The highest mean serum 25-OHD concentrations were found in 1992, mainly due to very high summer levels of that year, an observation that also holds true for the other years with mean serum 25-OHD concentrations above 40 nmol/l. Likewise, the

TABLE 3

Differences in average serum androstenedione (ng/ml) and 25-OHD (nmol/l) of paired pregnancy samples of Finnish women with specific pregnancy history[a]

Sampling Season	Summer–Summer	Summer–Winter	Winter–Summer	Winter–Winter
25-OHD, nmol/l (mean difference[b] with p-value)				
Summer–Summer		1.2(0.83)	−6.9(0.11)	16.6(0.001)[c]
Summer–Winter			−8.04(0.11)	17.8(0.001)[c]
Winter–Summer				9.7(0.02)[c]
Winter–Winter				
Androstenedione ng/mL (mean difference[b] with p-value)				
Summer–Summer				
Summer–Winter	−0.3(0.4)			
Winter–Summer	−0.2(0.5)	0.1(0.8)		
Winter–Winter	−0.2(0.6)	−0.6(0.1)	−0.5(0.2)	

[a]Abbreviation is as follows: 25-OHD, 25-hydroxy vitamin D.
[b]Mean differences between averages of paired serum.
[c]Mean 25-OHD concentrations for Winter–Winter paired samples are significantly lower than other season pairs.

lowest total mean 25-OHD concentrations were found in 1996, the year with the lowest summer 25-OHD concentrations. The year 1996 was the only year with average summer 25-OHD concentrations less than 35 nmol/l. The low summer values in 1996 are probably due to the cold weather in 1996 (below the average for the century) and the fact that July 1996 was the wettest July in 100 yr in parts of Finland (21). Thus, the possibility of obtaining 25-OHD from sunlight was lower compared to other years.

The diet of Finnish pregnant women has been fortified with 25-OHD since February 2003 (22). Our study samples were predominantly taken before this date. Differences in serum 25-OHD levels can therefore be attributed to seasonal variation. This implies that summer 25-OHD status, which is a reflection of exposure to UV light from the sun is the most important determinant of 25-OHD status in our Nordic cohort. This is because at latitudes above 35°C (such as Finland), winter sunlight cannot stimulate cutaneous production of pre-25-OHD since most, if not all, of the UVB photons below 315 nm are absorbed by the ozone layer (1). Our ability to detect the low summer serum 25-OHD values in the 1996 samples also reinforces the fact that 25-OHD is stable if stored properly at $-25°$C for many years, and possible deterioration is random.

For sample pairs with identical sampling seasons, average 25-OHD levels for the second pregnancy samples were higher than the first pregnancy levels for both winter–winter and summer–summer pairs. These results are consistent with previous findings that pregnancy increases nutrition awareness. Szwajcer et al. (23) carried out interviews of 1-h duration with 60 women categorized in 5 groups: nulliparous, nonpregnant women; groups of first, second, and third trimester pregnant nulliparous women; and second pregnancy women in the first trimester. They found that pregnancy leads to increased nutritional awareness, which becomes a habit during the second pregnancy. The increased health conscience among pregnant women may lead to frequent sunlight exposure or increased dietary intake of foods rich in vitamin D, which could translate into higher levels of average 25-OHD for women with second pregnancies compared to the women with first pregnancies.

The androstenedione levels were somewhat higher in winter than in summer. We found this also in the maternity cohort samples used in Holl et al. study (24) (data not shown). These results are consistent with experimental studies (25,26). Sing and Krishna (25) determined seasonal changes in testosterone and androstenedione levels in adult rats and observed the highest androstenedione levels in winter (November–January). In an earlier study, Smith et al. (26) studied the effect of seasonal variability on the levels of androstenedione and testosterone in male blue foxes. They observed a steady increase in the levels of both hormones during winter months (November–March). Contrary to our findings, Bjørnerem et al (11) and Brambilla et al. (27) did not observe any seasonal variation in the serum androgens (DHEA and testosterone) in blood samples of adult males and females. DHEA is a precursor in synthesis of androstenedione,

which in turn is reversibly converted to testosterone (8). Thus, seasonal stability in serum levels of androstenedione should correlate with the levels of DHEA and testosterone.

In prospective epidemiologic studies using biomarkers as quantitative estimates of past exposures and disease risk, serial samples may provide a more reliable estimate compared to static, single sample, estimates (28). Although there is compelling evidence on the difference between summer and winter levels of 25-OHD, to the best of our knowledge, no study has compared serial (paired) samples taken both in similar and different seasons. Our study shows that significant differences in 25-OHD levels due to sampling season exist also for paired samples, especially if the samples are drawn in winter compared to other seasons. We suggest that epidemiologic studies assessing 25-OHD levels and disease risk should appropriately stratify and match subparts by sampling season and use serial samples wherever possible to get better insight into the vitamin D status of cases and controls.

REFERENCES

1. Holick MF: Vitamin D: importance in the prevention of cancer, Type 1 diabetes, heart disease, and osteoporosis. *Am J Clin Nutr* **79,** 362–371, 2004.
2. Webb AR, Kline L, and Holick MF: Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. *J Clin Endocrinol Met* **67,** 373–378, 1988.
3. Lips P: Vitamin D physiology. *Prog Biophys Mol Biol* **92,** 4–8, 2006.
4. Wolpowitz D and Gilchrest BA: The vitamin D questions: how much do you need and how should you get it. *J Am Acad Dermatol* **54,** 301–317, 2006.
5. Holick MF: Vitamin D deficiency. *N Engl J Med* **357,** 266–281, 2007.
6. Antonuicci DM, Black DM, and Sellmeyer DE: Serum 25-Hydroxyvitamin is unaffected by multiple freeze–thaw cycles. *Clin Chem* **51,** 258–261, 2005.
7. Ocke MC, Shrijver J, Obermann-De Boer GL, Bloemberg BP, Haenen GR, et al: Stability of blood (pro)vitamins during 4 years of storage at $-20°$C: consequences for epidemiologic research. *J Clin Epidemiol* **48,** 1077–1085, 1995.
8. Bodnar LM, Catov JM, Wisner KL, and Klebanoff MA: Racial and seasonal differences in 25-hydroxyvitamin D detected in maternal sera frozen for over 40 years. *Br J Nutr* **23,** 1–7, 2008.
9. Ming-qiu HU and Yan-qun NA: Metabolism of adrenal androgen and its impacts on prostate cancer after castration. *Chin Med J* **121,** 369–374, 2008.
10. Svartberg J, Jorde R, Sundsfjord J, Bønaa KH, and Barrett-Connor E: Seasonal variation of testosterone and waist to hip ratio in men: the Tromsø Study. *J Clin Endocrinol Metab* **88,** 3099–3104, 2003.
11. Bjørnerem Å, Straume B, Øian P, and Berntsen GK: Seasonal variation of estradiol, follicle stimulating hormone, and dehydroepiandrosterone sulfate in women and men. *J Clin Endocrinol Metab* **91,** 3789–3802, 2006.
12. Andersson AM, Carlsen E, Petersen JH, and Skakkebaek NE: Variation in levels of serum inhibin B, testosterone, estradiol, luteinizing hormone, follicle-stimulating hormone, and sex hormone-binding globulin in monthly samples from healthy men during a 17-month period: possible effects of seasons. *J Clin Endocrinol Metab* **88,** 932–937, 2003.
13. Maes M, Mommen K, Hendrickx D, Peeters D, D'Hondt P, et al.: Components of biological variation, including seasonality, in blood concentrations of TSH, TT3, FT4, PRL, cortisol and testosterone in healthy volunteers. *Clin Endocrinol* **46,** 587–598, 1997.

14. Lou Y-R and Tuohimaa P: Androgen enhances the anti-proliferative activity of 25-OHD$_3$by suppressing 24-hydoxylase expression in LNCaP cells. *J Steroid Biochem Mol Biol* **92,** 307–315, 2004.

15. Zhao X-Y, Peehl, DM, Navone NM, and Feldman D: 1 alpha, 25-dihydroxy vitamin D3 inhibits prostate cancer cell growth by androgen dependent and androgen independent mechanisms. *Endocrinology* **141,** 2548–2556, 2000.

16. Leman ES, Arlotti JA, Dhir R, and Getzenberg RH: 25-OHD and androgen regulation of prostatic growth. *J Cell Biochem* **90,** 138–147, 2003.

17. Koskela P, Anttila T, Bjørge T, Brunsvig A, Dillner J, et al.: Chlamydia trachomatis infection as a risk factor for invasive cervical cancer. *Int J Cancer* **85,** 35–39, 2000.

18. Holl K, Lundin E, Kaasila M, Grankvist K, Afanasyeva Y, et al.: Effect of long-term storage on hormone measurements in samples from pregnant women: the experience of the Finnish Maternity Cohort. *Acta Oncol* **47,** 406–412, 2008.

19. Nakamura K, Nashimoto M, and Yamamoto M: Summer/winter differences in the serum 25-hydroxyvitamin D$_3$ and parathyroid hormone levels of Japanese women. *Int J Biometeorol* **44,** 186–189, 2000.

20. Harris SS and Dawson-Hughes B: Seasonal changes in plasma 25-hydroxyvitamin D concentrations of young American black and white women. *Am J Clin Nutr* **67,** 1232–1236, 1998.

21. Kløve B and Bengtsson L: Runoff generation in a plough-drained cutover fen in central Finland. *J Hydrology* **218,** 157–168, 1999.

22. Laaksi IT, Ruohola JP, Ylikomi TJ, Auvinen A, Haataja RI, et al.: 25-OHD fortification as public health policy: significant improvement in 25-OHD status in young Finnish men. *Eur J Clin Nutr* **60,** 1035–1038, 2006.

23. Szwajcer EM, Hiddink GJ, Maas L, Koelen MA, and van Woerkum CMJ: Nutrition-related information-seeking behaviors of women trying to conceive and pregnant women: evidence for the life course perspective. *Fam Pract* **25**, 99–104, 2008.

24. Holl K, Lundin E, Surcel HM, Grankvist K, Koskela P, et al.: Endogenous steroid hormone levels in early pregnancy and risk of testicular cancer in the offspring: a nested case-referent study. *Int J Cancer* **124,** 2923–2928, 2009.

25. Singh UP and Krishna A: Seasonal changes in circulating testosterone and androstenedione concentration and their correlation with the anomalous reproductive pattern in the male Indian sheath-tailed bat, *Taphozous longimanus*. *J Exp Zool* **287,** 54–61, 2000.

26. Smith AJ, Mondain-Monval M, Møller OM, Scholler R, and Hansson V: Seasonal variations of LH, prolactin, androstenedione, testosterone and testicular FSH binding in the male blue fox (Alopex lagopus). *J Reprod Fertil* **74,** 449–458, 1995.

27. Brambilla DJ, O'Donnell AB, Matsumoto AM, and McKinlay JB: Lack of seasonal variation in serum sex hormone levels in middle-aged to older men in the Boston area. *J Clin Endocrinol Metab* **92,** 4224–4429, 2007.

28. Woltering EA, Hilton RS, Zolfoghary CM, Thomson J, Zietz S, et al.: Validation of serum versus plasma measurements of chromogranin a levels in patients with carcinoid tumors: lack of correlation between absolute chromogranin a levels and symptom frequency. *Pancreas* **33,** 250–254, 2006.

Short Communication

Serum 25-hydroxyvitamin D at pregnancy and risk of breast cancer in a prospective study

Calypse B. Agborsangaya [a,b,*], *Helja-Marja Surcel* [b], *Adetunji T. Toriola* [a,b], *Eero Pukkala* [c], *Seppo Parkkila* [d], *Pentti Tuohimaa* [d], *Annekatrin Lukanova* [e], *Matti Lehtinen* [a,b]

[a] Tampere School of Public Health, University of Tampere, Finland
[b] National Institute for Health and Welfare, Oulu, Finland
[c] Finnish Cancer Registry, Institute for Statistical and Epidemiological Cancer Research, Helsinki, Finland
[d] Department of Anatomy, University of Tampere and Tampere University Hospital, Finland
[e] Department of Cancer Epidemiology, German Cancer Research Centre, Heidelberg, Germany

A B S T R A C T

Background: Several laboratory and epidemiological studies have inversely linked endogenous vitamin D and the risk of breast cancer. The acquisition of vitamin D over time on the relative risk (RR) of the disease development is not known. In a longitudinal study, we evaluated the association between vitamin D levels at pregnancy over time with the risk of breast cancer, and pregnancy-associated breast cancer.

Method: The risk for subsequent development of breast cancer associated with serum 25-hydroxyvitamin (25-OHD) levels was assessed for consecutive (1st and 2nd pregnancy) samples of 100 cases, with mean lag times (μ_t) of 7.4 and 4.6 years between sampling and the diagnosis, and matched (parity, age, year, season) controls. Pregnancy-associated breast cancer (PABC, 111 case–control pairs, $\mu_t = 1$year) risk was also studied. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated using the lowest quintile as the reference.

Results: Serum 25-OHD level was not associated with an increased risk neither at the 1st nor at the 2nd pregnancy samples (OR = 1.4, 95%CI 0.6–3.4; OR 1.4, 95%CI 0.7–2.8, respectively), but was associated with an increased risk of PABC (OR = 2.7, 95%CI 1.04–6.7).

Conclusion: Generally, vitamin D may not be related to breast cancer risk but the increased PABC risk fits the association of vitamin D with the most aggressive cancers, and warrants caution with vitamin D supplementation during pregnancy.

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1. Introduction

Vitamin D can inhibit breast cancer cell proliferation as well as promote apoptosis and cell differentiation in normal and malignant breast tissues.[1–4] In post-menopausal women, low levels of 25-OHD are associated with an increased risk of breast cancer.[5,6] Although a similar observation has been made in premenopausal women,[7] the opposite may be true.[5,8]

Levels of vitamin D, a pro-hormone primarily synthesised via exposure to ultraviolet B (UVB) radiation from sunlight, are subject to great variation over time. However, no study with prediagnostic serial samples has evaluated the acquisition of vitamin D and relative risk for the subsequent development of breast cancer.

We conducted a prospective nested case–control study within the Finnish Maternity Cohort (FMC), the world's largest serum bank for female donors,[9] to evaluate the risk of breast cancer associated with vitamin D (as measured by 25-OHD) levels at the 1st and the 2nd pregnancy. We also determined the relationship between 25-OHD levels and risk of pregnancy-associated breast cancer (PABC, diagnosed within one year of delivery). Since 1983, virtually all (>98%) Finnish women (altogether 750,000 women) have donated 1st trimester serum samples to the FMC at each pregnancy.[9] Cases and controls were ascertained by the Finnish Cancer Registry (FCR).[10] The study was approved by the local ethical committee.

2. Materials and methods

We identified 100 cases with at least two singleton pregnancies (follow-up time $\leqslant 10$ years) before the diagnosis of breast cancer, and matched them to cancer-free controls for season of blood withdrawal [summer (May–August) or winter (December–March)], sampling age (± 1 year), sampling year and parity (± 1). Stratification by age at diagnosis as previously defined[8] did not identify post-menopausal breast cancer cases.

In addition, all PABC cases (111 in total) were identified and matched with controls for age (± 1 year), parity (± 1) and date of index blood sampling (± 15 d). The cases were matched with the controls on a one-to-one basis.

25-OHD was measured by 25-OHD IDS-radioimmunoassay (RIA) from IDS Ltd., Boldon, UK. Mean coefficients of variations were 2.0% at 28.0 nmol/l (intra-assay) and 2.8% at 26.7 nmol/l (inter-assay). Lab analyses were done blinded using a single batch of the assay kits. Mean differences in 25-OHD levels were compared by the Mann–Whitney non-parametric test. Quintile cut-off points for the analysis were determined using 25-OHD quintiles levels of the controls. These quintiles were then used to estimate the relative risk (expressed as odds ratio (OR)) of breast cancer at 95% confidence intervals (CI) using multivariate conditional logistic regression. The multivariate model was adjusted for gestational day at sampling. Trend analyses were performed using a linear-by-linear association chi-square statistics on ORs directly. Separate analyses were performed for the first and second pregnancy levels. The risk of breast cancer was also evaluated by comparing the lowest quintile to the other quintiles combined. A two-sided p value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 15 for windows (SPSS Inc., Chicago, IL).

3. Results

With the exception of time-lag between sampling and cancer diagnoses, the study groups were comparable (Table 1). We also found no major differences in the 25-OHD levels between cases and controls for the 1st or the 2nd pregnancy samples (mean difference, $\mu_t = 0.09$, $p = 0.7$; $\mu_t = 8.0$, $p = 0.1$, respectively). Using the lowest quintile as the reference, we found no significant association of serum 25-OHD levels with the relative risk of breast cancer quintile by quintile (Table 2), or other (2nd to 5th) quintiles combined, neither for the 1st (OR = 1.4, 95%CI 0.6–3.4) nor for the 2nd pregnancy samples (OR = 1.4, 95%CI 0.7–2.9), Table 2). On the contrary, higher levels of vitamin D (2nd to 5th quintiles combined against the lowest quintile) were associated with an increased risk of PABC (OR = 2.7, 95%CI 1.04–6.8). The PABC-associated risk was two- to four-fold higher in the different quintiles of vitamin D levels compared to the reference (Table 2). Adjustment for gestational day had no material effect on the results.

4. Discussion

This is the first study to look at vitamin D during pregnancy in relation to the risk of subsequent development of breast cancer. The breast cancer cases diagnosed among the donors of the Finnish Maternity Cohort were premenopausal, and the null association found in our paired (pregnancy) sample material is in line with a recent study on premenopausal breast cancer showing no association between serum vitamin D levels and risk of breast cancer.[5] A null association was also observed in older women in the large case–control study nested within the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer trial.[8] Our study adds to these findings since even the over time assessment of vitamin D acquisition did not disclose any significant risk associated with premenopausal breast cancer.

It is intriguing that higher levels of serum 25-OHD were associated with a two- to fourfold increased risk of PABC, the more aggressive sub-entity of breast cancer.[11] This is also the first longitudinal study to evaluate the relationship between vitamin D levels and PABC. Although the results contradict experimental evidence,[1–4] they are in line with studies on the association of increased 25-OHD levels and elevated risk of aggressive prostate cancer.[12,13]

Levels of vitamin D binding protein (VDBP) are significantly increased during pregnancy.[14] It is the carrier protein of vitamin D in circulation, with higher affinity for 25-OHD than for 1, 25-$(OH)_2D$,[15] which probably favours increased relative availability of the biologically more active, anti-carcinogenic form, 1, 25-$(OH)_2D$. Our observation might reflect an inverse association between the free levels of the two forms of vitamin D. On the other hand, higher 25-OHD levels within the body may affect its metabolism, e.g. increased 24-hydroxylation,[16] and lead to reduced tissue concentrations of 1, 25-$(OH)_2D$, with consequent low anti-proliferative activity again.

In conclusion, the findings from our study do not support the hypothesis that prediagnostic circulating vitamin D levels are associated with risk of premenopausal breast cancer. We, however, observed the possibility that higher levels of vitamin D may be associated with increased risk of breast cancer arising soon after pregnancy. Further, independent studies on vitamin D and risk of pregnancy-associated breast cancer are warranted. We propose that recommendations for dietary

Table 1 – Baseline characteristics of breast cancer cases and controls with consecutive samples and pregnancy-associated breast cancer cases and controls of the Finnish Maternity Cohort.

	1st pregnancy samples		2nd pregnancy samples		PABC[a]	
	Cases	Controls	Cases	Controls	Cases	Controls
Number (N)	100	100	100	100	111	111
Mean age[b]	30.5	30.5	33.3	33.3	34.4	34.4
Gestational day (d)	73.4	73.5	78.7	77.1	77.9	
Parity	2.0	2.1	3.1	3.4	2.0	2.3
Follow-up time[b]	7.4		4.6		1	
Age at diagnosis (range)	38.0 (25.1–50.0)				35.6 (24.9–43.9)	

a Pregnancy-associated breast cancer (diagnosed within 1 year of sampling).
b Calculated in years.

Table 2 – Relative risks (odds ratio (OR) with 95% confidence interval, CI) of breast cancer and pregnancy-associated breast cancer by quintiles of serum vitamin D concentrations.

25-(OH)D	Cases	Controls	OR (95%CI)	p Value	p_{trend}
First pregnancy					
Quintiles[c]					
Q_1[a]	16	19	1.0		
Q_2	23	20	1.5 (0.5–2.0)	0.5	
Q_3	19	20	1.2 (0.4–3.6)	0.8	
Q_4	19	20	1.2 (0.4–3.7)	0.7	
Q_5	23	21	1.4 (0.5–4.2)	0.6	0.4
Q_{2-5}	84	81	1.4 (0.6–3.4)	0.5	
Second pregnancy					
Quintiles[d]					
Q_1[a]	16	21	1.0		
Q_2	18	21	1.0 (0.4–2.6)	1.0	
Q_3	26	20	1.7 (0.7–4.2)	0.2	
Q_4	11	20	0.7 (0.2–1.9)	0.7	
Q_5	29	18	2.1 (0.8–5.1)	0.1	0.5
Q_{2-5}	84	79	1.4 (0.7–2.8)	0.4	
PABC[b] subjects					
Quintiles[e]					
Q_1[a]	12	22	1.0		
Q_2	29	23	2.9 (1.0–8.4)	0.05	
Q_3	21	23	2.0 (0.7–6.0)	0.2	
Q_4	31	21	3.7 (1.2–11.8)	0.03	
Q_5	18	22	1.9 (0.5–6.7)	0.3	0.4
Q_{2-5}	99	89	2.7 (1.04–6.7)	0.04	

a The lowest (reference) quintile of serum 25-hydroxy vitamin D.
b Pregnancy-associated breast cancer (follow-up time up to 1 year following pregnancy).
c Quintiles(⩽27.5[a], 27.6–37.5, 37.6–48.5, 46.6–55.5, ⩾61.6 nmol/l).
d Quintiles (⩽27.8, 27.9–36.9, 37.0–46.5, 46.6–55.5, ⩾56.6 nmol/l).
e Quintiles (⩽25.8, 25.9–34.9, 35.0–44.7, 44.8–64.0, ⩾64.1 nmol/l).

supplementation of vitamin D during pregnancy are reconsidered.

Conflict of interest statement

None declared.

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REFERENCE

1. Welsh J, Wietzke JA, Zinser GM, et al. Vitamin D-3 receptor as a target for breast cancer prevention. *J Nutr* 2003;**133**:2425–33.
2. Lowe L, Hansen CM, Senaratne S, Colston KW. Mechanisms implicated in the growth regulatory effects of vitamin D compounds in breast cancer cells. *Recent Results Cancer Res* 2003;**164**:99–110.

3. Colston KW, Hansen CM. Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. *Endocr Relat Cancer* 2002;**9**:45–59.

4. Eisman JA, MacIntyre I, Martin TJ, Frampton RJ, King RJ. Normal and malignant breast tissue is a target organ for 1, 25-$(OH)_2$ vitamin D3. *Clin Endocrinol (Oxford)* 1980;**13**:267–72.

5. Crew KD, Gammon MD, Steck SE, et al. Association between plasma 25-hydroxyvitamin D and breast cancer risk. *Cancer Prev Res* 2009;**2**:598–604.

6. Abbas S, Linseisen J, Slanger T, et al. Serum 25-hydroxyvitamin D and risk of post-menopausal breast cancer – results of a large case–control study. *Carcinogenesis* 2008;**29**:93–9.

7. Abbas S, Chang-Claude J, Linseisen J. Plasma 25-hydroxyvitamin D and premenopausal breast cancer risk in a German case–control study. *Int J Cancer* 2009;**124**:250–5.

8. Freedman DM, Chang SC, Falk RT, et al. Serum levels of vitamin D metabolites and breast cancer risk in the prostate, lung, colorectal and ovarian cancer screening trial. *Cancer Epidemiol Biomarkers Prev* 2008;**17**:889–94.

9. Lyytikäinen E, Kaasila M, Koskela P, et al. Chlamydia trachomatis seroprevalence atlas of Finland 1983–2003. *Sex Transm Infect* 2008;**84**:19–22.

10. Teppo L, Pukkala E, Lehtonen M. Data quality and quality control of a population-based cancer registry. Experience in Finland. *Acta Oncol* 1994;**33**:365–9.

11. Rodriguez AO, Chew H, Cress R, et al. Evidence of poorer survival in pregnancy-associated breast cancer (abstract only). *Obstet Gynecol* 2008;**112**:71–8.

12. Ahn J, Peters U, Albanes D, et al. Serum vitamin D concentration and prostate cancer risk: a nested case–control study. *J Natl Cancer Inst* 2008;**100**:796–804.

13. Tuohimaa P, Tenkanen L, Ahonen M, et al. Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a longitudinal, nested case–control study in the Nordic countries. *Int J Cancer* 2004;**108**:104–8.

14. Haddad JG. Plasma vitamin D-binding protein (Gc-globulin): multiple tasks. *J Steroid Biochem Mol Biol* 1995;**53**:1–6.

15. Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P. Influence of the vitamin D-binding protein on the serum concentration of 1, 25-dihydroxyvitamin D3. Significance of the free 1, 25-dihydroxyvitamin D3 concentration. *J Clin Invest* 1981;**67**:589–96.

16. Welsh JE. Vitamin D and breast cancer: insights from animal models. *Am J Clin Nutr* 2004;**80**:1721–4.

Association between Epstein–Barr virus infection and risk for development of pregnancy-associated breast cancer: Joint effect with vitamin D?

Calypse B. Agborsangaya [a,b,*], Tuula Lehtinen [c], Adetunji T. Toriola [a,b], Eero Pukkala [d], Helja-Marja Surcel [b], Rosamaria Tedeschi [e], Matti Lehtinen [a]

[a] Tampere School of Public Health, University of Tampere, Tampere, Finland
[b] National Institute for Health and Welfare, Oulu, Finland
[c] Department of Clinical Oncology, Tampere University Hospital, Tampere, Finland
[d] Finnish Cancer Registry, Institute for Statistical and Epidemiological Cancer Research, Helsinki, Finland
[e] Microbiology–Immunology and Virology Unit, Centro di Riferimento Oncologico, Istituto Di Ricovero e Cura a Carattere Scientifico, Aviano, Italy

A R T I C L E   I N F O

A B S T R A C T

Background: Few studies have evaluated the role of the ubiquitous Epstein–Barr virus (EBV) infection, together with levels of the immunomodulator, vitamin D, in different breast cancer entities. We studied, prospectively, the association of EBV and vitamin D status with the risk of pregnancy-associated breast cancer (PABC), breast cancer diagnosed during pregnancy or 1 year post-partum, using a nested case–control study.
Methods: Serum vitamin D and antibodies to EBV were measured for 108 PABC cases of the Finnish Maternity Cohort, and 208 controls matched for date of birth, date of sampling and parity. The joint effect of vitamin D and EBV on the risk of PABC was evaluated.
Results: EBV seropositivity was generally not associated with the risk of PABC. Among individuals with sufficient (⩾75 nmol/l) levels of vitamin D, we, however, found similar increased risk estimates for PABC associated with serum immunoglobulin G (IgG) antibodies to EBV early antigens [odds ratio (OR) = 7.7, 95% (confidence interval) CI 1.4–42.3] and the viral reactivator protein, ZEBRA (OR = 7.8, 95% CI 1.1–61.2).
Conclusion: Immunological markers of EBV reactivation status among individuals with sufficient vitamin D levels were consistently associated with increased risk of the disease. This suggests that EBV reactivation may be an indicator of the progression of breast cancer occurring soon after pregnancy, while the virus probably is not the aetiological agent.

1. Introduction

Established risk factors of breast cancer explain only about 50% of the disease aetiology.[1] This has prompted interest on environmental agents, including viral infections in breast carcinogenesis. Epstein–Barr virus (EBV), noted as a class 1 carcinogen over a decade ago by the International Agency for Research on Cancer (IARC),[2] has been of particular interest.[3–5]

* Corresponding author: Address: Tampere School of Public Health, Sorinkatu 1, 33100 Tampere, Finland. Tel.: +358 401901671; fax: +358 335518057.
E-mail address: calypse.agborsangaya@uta.fi (C.B. Agborsangaya).

The hypothesis that EBV is involved in the aetiology of breast cancer was originally based on evidence that mothers with latent EBV infection shed the virus in their breast milk.[6] On the other hand, lymphoepithelioma-like carcinomas such as some gastric cancers have long been known to be positive for EBV,[7] and known breast cancer subtypes portray comparable features with lymphocytic infiltration. EBV DNA has been identified in benign breast tissue samples of both immuno-suppressed and immunocompetent individuals[8] and EBV-positive lymphoma growth has been described also in the breast tumours.[9]

The detection rate of EBV in breast carcinoma cells varies. Some studies report the absence of the EBV genome,[10–12] while others report detection rates between 20% and 60%.[13–15] The disparate findings have been attributed to methodological variation.[16] Serological studies have evaluated the association between EBV antibodies and the risk of breast cancer, albeit also with controversial results.[3–5] These findings suggest that the role of EBV in a sub-group of breast cancer aetiology may be either causal or that of a bystander in conjunction with EBV reactivation.

Pregnancy-associated breast cancer (PABC) is diagnosed most often during pregnancy or 1 year post-partum. The incidence of PABC is low (1–3 in 10,000 pregnancies), but has been increasing,[17] as women elect to delay childbearing to their third and fourth decades. PABC not infrequently has lympho-epithelial vascular invasion,[18,19] probably involving also EBV-positive cells. We evaluated the association between pre-existing EBV infection and the risk of PABC.

EBV reactivation takes place not infrequently during pregnancy.[20,21] Following EBV reactivation, up-regulation of viral oncogenes and cellular growth regulating genes, such as the latent membrane protein (LMP1), takes place. Also, vitamin D status modulates the immune response to and reactivation of EBV, and interplay of the two has recently been suggested to play a role in chronic disease (multiple sclerosis) development,[22,23] often following pregnancy. It is possible that EBV reactivation, by vitamin D levels, is associated with risk of breast cancer.

In a previous study, we showed that higher vitamin D levels are associated with an increased risk of PABC.[24] This study further evaluates the association between serum antibodies indicating EBV infection or reactivation and the risk of subsequent development of PABC in a case–control study nested within the Finnish Maternity Cohort (FMC) involving females with adequate or inadequate supply of vitamin D.

2. Materials and methods

2.1. Study population

The Finnish Maternity Cohort (FMC) is a serum repository of over 1.6 million pregnant women serum samples established in 1983 in Finland, with a national coverage among pregnant women of approximately 98%. The samples are withdrawn at municipal maternity care units during the first trimester of pregnancy (gestational weeks 10–12), following an informed consent, for screening of congenital infections. After the screening, 1–3 mL volume of serum is stored at –25 °C in poly-propylene cryo vials at the National Institute for Health and Welfare, Oulu, Finland.[25]

Cases and controls for this study were derived from a linkage of the FMC and the Finnish Cancer Registry (FCR), the national population-based cancer register in Finland. The FCR established in 1953 has a national coverage which is virtually complete with no loss to follow-up.[26] The record-linkage was done at the FCR using unique personal identity codes given to all residents in Finland.

Our study material constituted 111 incidence density sampled PABC cases–control pairs that were previously used.[24] Three case samples were excluded because of insufficient sample volume. Each case was matched to 2 controls for date of birth (±1 year), time of serum sampling (±14 days) and parity (±1). Parity was defined as the number of pregnancies at the time of sample withdrawal. A total of 208 controls were available for analysis. Data for serum 25-OHD for all samples were available from our previous analysis,[24] and were used to determine joint effects of vitamin D and EBV past infection. The study was approved by the ethical committee of the National Institute for Health and Welfare, Finland.

2.2. Laboratory analyses

Maternal immunoglobulin G (IgG) antibodies to EBV nuclear antigen (EBNA) and early antigen (EA) were assessed by commercial enzyme-linked immunosorbent assays (ELISAs) based on recombinant proteins (Biotest AG, Frankfurt, Germany). Cut-off values, defining positive or negative samples on all plates, were calculated by following the manufacturer's recommendations. Samples that were EBNA negative were also assessed for VCA IgG to evaluate past infection. In addition, EBV Zebra IgG antibodies were evaluated by an in-house-made peptide-based ELISA, as previously described.[27,28] Laboratory analyses were performed on coded samples with case–control status masked.

2.3. Statistical analysis

Descriptive statistics were calculated for cases and controls. Relative risks (RR) expressed as odds ratio (ORs) with 95% confidence intervals (CI) were estimated by conditional logistic regression for the matched case–control triplets using SPSS 15 for windows (SPSS Inc., Chicago, IL). After categorising by sufficient levels of vitamin D (75 nmol/L of serum 25-OHD was used as the cut-off value[29]), the matching criteria were digressed. Thus, unconditional logistic regression was used to calculate the risk estimates for the serological EBV markers and PABC risk, adjusting for the matching criteria. A two-sided p value <0.05 was considered statistically significant.

3. Results

Mean age at serum sampling for the PABC cases was more than that of the pregnant women in Finland (34.4 years versus 31.5 years (Table 1)). Almost all the samples were positive for EBNA IgG antibodies (95.4% and 96.3% for cases and controls, respectively). Seventy-three percent and 12% of the cases were positive for EBV EA and ZEBRA IgG antibodies,

Table 1 – Baseline characteristics of pregnancy-associated breast cancer cases and controls samples of the Finnish Maternity Cohort (FMC).

	Cases	Controls
Number (N)	108	208
Mean age[a]	34.2	34.4
Gestational day (d)	77.1	77.9
Parity	2.0	2.3
Follow-up time[a]		1
Age at diagnosis (range)	35.6 (24.9–43.9)	

[a] Calculated in years

Table 2 – Relative risk odds ratios (OR) with 95% confidence intervals (CI) for pregnancy-associated breast cancer in relation to the detection of positive immunoglobulin G (IgG) antibodies to Epstein–Barr virus (EBV) antigens in the Finnish Maternity Cohort.

Seropositivity	Number of			
	Cases	Controls	ORs	95% CI
EBNA				
No	6	8	1	
Yes	102	200	0.7	0.2–2.2
EA				
No	30	73	1	
Yes	78	135	1.5	0.9–2.5
ZEBRA				
No	94	191	1	
Yes	14	17	1.4	0.7-2.8

respectively, compared to 64% and 8.9% of the controls (Table 2). Seventy-seven percent (76.9%) of the cases were positive for serological markers of EBV reactivation (anti-EA or anti-ZEBRA) compared to 66.8% for controls.

The concentrations of anti-EA IgG and anti-ZEBRA IgG were significantly correlated ($p < 0.04$). There was no association between the risk of PABC and EBV antibodies to the presence of EBNA, EA and ZEBRA antigens (Table 2). Individuals positive for either anti-EA IgG or anti-ZEBRA IgG antibodies had a somewhat increased risk of PABC with borderline statistical significance (OR = 1.7, 95% CI 1.0–2.8).

Among individuals with sufficient levels of serum 25-OHD ($\geqslant 75$ nmol/l), we noted a significantly increased risk of PABC associated with positivity for both serum EA IgG antibodies (OR = 7.7, 95% CI 1.4–42.3) and ZEBRA IgG antibodies (OR = 7.8, 95% CI 1.1–61.2) (Table 3). To increase the sensitivity for the determination of EBV reactivation, we considered positivity for either EA or ZEBRA IgG antibodies. The presence of either EA or ZEBRA IgG antibodies was significantly associated with the risk of PABC (OR = 12.1 95% CI 1.3–107.3). No such observations were made among individuals with less than the sufficient levels of vitamin D (Table 3).

4. Discussions

In general, we found no association between pre-existing EBV infection and the risk of development for pregnancy-associated breast cancer. Positivity for immunological markers of EBV reactivation among individuals with sufficient levels of vitamin D was, however, associated with a significantly increased risk of breast cancer occurring during or soon after pregnancy.

Over 96% of the controls were EBV seropositive, comparable to previous data from pregnant women populations in Finland[30] and the United States of America.[20] The finding that PABC cases were older at serum withdrawal compared to other women in our cohort is consistent with the increasing

Table 3 – Odds ratios(OR) with 95% confidence intervals (CI) for pregnancy-associated breast cancer and EBV EBNA, EA and ZEBRA IgG antibody positivity among women with sufficient and less than sufficient levels of vitamin D in first trimester serum samples of the Finnish Maternity Cohort.

Seropositivity		Number of			
		Cases	Controls	OR[a]	95% CI
Vitamin D (<75 nmol/l)					
EBNA	No	6	8	1	
EBNA	Yes	89	176	0.9	0.2–3.5
EA	No	28	60	1	
EA	Yes	68	124	0.8	0.4–1.6
ZEBRA	No	85	169	1	
ZEBRA	Yes	10	15	1.5	0.5–4.9
Vitamin D (≥75 nmol/l)					
EBNA	No	0	0		
EBNA	Yes	13	24	n.a.	
EA	No	2	14	1	
EA	Yes	11	10	7.7	1.4 – 42.3
ZEBRA	No	9	22	1	
ZEBRA	Yes	4	2	7.8	1.1 – 61.2

Vitamin D levels defined as insufficient (serum 25-OHD <75 nmol/l) and sufficient (serum 25-OHD ≥75 nmol/l).
n.a = not available.
[a] Adjusted for age and season of sampling.

incidence of PABC in women postponing childbearing to later ages. EBV reactivation, however, is thought to be independent of the maternal age at pregnancy.[20]

The null association between past EBV infection and the risk for subsequent development of PABC in this study is consistent with a previous study on EBV and premenopausal breast cancer.[3] In that cross-sectional study, no association was found between positivity for EBV and the risk of breast cancer in young women. In another study,[4] the authors observed that the mean anti-EBNA IgG antibody levels were significantly higher in breast cancer cases than in controls with benign breast disease. In a recent case–control study nested within the Janus Serum Bank cohort,[5] EBV IgG antibody levels in serum samples drawn at least 4 years before breast cancer diagnosis were not associated with the risk of the disease.

This is the first study, to our knowledge, to evaluate the interplay between prediagnostic EBV reactivation and vitamin D with the risk of PABC. Only 12% of our cases and controls had sufficient levels of vitamin D, as vitamin D insufficiency is very common in pregnant women.[31,32] Positivity for both serum anti-EBV ZEBRA and anti-EBV EA IgG antibodies indicated the occurrence of EBV reactivation, almost exclusively in individuals with sufficient levels of vitamin D. These women, who were positive for anti-EBV EA or ZEBRA IgG antibodies, had a significantly increased risk for PABC.

The active form of vitamin D, dihydroxyvitamin D_3, is a potent immunomodulator and may modulate the immune response to EBV infection by suppressing T-cell proliferation.[22,23] It is possible that higher levels of vitamin D reduce the immune surveillance of EBV, thus promulgating viral reactivation from EBV harbouring B cells. It is equally possible that EBV reactivation was caused by the true cause of PABC and just became detectable in individuals with sufficient vitamin D status.

The discrepant results on the association between breast cancer and EBV infection in previous studies, which are based on EBV serological markers, are probably due to the differences in the serological markers of EBV infection used and the heterogeneity of the breast cancer cases. Methodological variation in detecting EBV has also been suggested to explain the results.[16] In this study, we employed four serological markers of EBV infection and found that the expression of EBV early antigens consistently suggested the reactivation of EBV in a proportion of PABC cases. The longitudinal and population-based nature of sample collection and cancer registration minimises the possibilities that our results are due to reverse causality bias or chance introduced because of case misclassification. Finally, it is possible that breast cancer heterogeneity may have diluted the association between past EBV infection and the risk of PABC, particularly because sub-groups were not categorised by hormone receptor status. The small sample size of this study, in particular, warrants further studies.

In conclusion, we found no association between past EBV infection and the subsequent risk of PABC. However, EBV reactivation among individuals with sufficient vitamin D levels was associated with increased risk of the disease. EBV reactivation may be an indicator of the progression (not an aetiological agent) of breast cancer occurring during pregnancy.

Conflict of interest statement

None declared.

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REFERENCES

1. Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. Proportion of breast cancer cases in the United States explained by well-established risk factors. *J Natl Cancer Inst* 1995;**87**:1681–5.
2. Griffin BE. Epstein–Barr virus (EBV) and human disease: facts, opinions and problems. *Mutat Res* 2000;**462**:395–405.
3. Richardson AK, Cox B, McCredie MR, et al. Cytomegalovirus, Epstein–Barr virus and risk of breast cancer before age 40 years: a case–control study. *Br J Cancer* 2004;**90**:2149–52.
4. Joshi D, Quadri M, Gangane N, Joshi R, Gangane N. Association of Epstein Barr virus infection (EBV) with breast cancer in rural Indian women. *PLoS One* 2009; 4:e8180. doi: 10.1371/ journal.pone.0008180.
5. Cox B, Richardson A, Graham P, et al. Breast cancer, cytomegalovirus and Epstein–Barr virus: a nested case–control study. *Br J Cancer* 2010;**102**:1665–9.
6. Junker AK, Thomas EE, Radcliffe A, et al. Epstein–Barr virus shedding in breast milk. *Am J Med Sci* 1991;**302**:220–3.
7. Iezzoni JC, Gaffey MJ, Weiss LM. The role of Epstein–Barr virus in lymphoepithelioma-like carcinomas. *Am J Clin Pathol* 1995;**103**:308–15.
8. Kleer CG, Tseng MD, Gutsch DE, et al. Detection of Epstein–Barr virus in rapidly growing fibroadenomas of the breast in immunosuppressed hosts. *Mod Pathol* 2002;**15**:759–64.
9. Abhyankar SH, Chiang KY, McGuirk JP, et al. Late onset Epstein–Barr virus-associated lymphoproliferative disease after allogeneic bone marrow transplant presenting as breast masses. *Bone Marrow Transplant* 1998;**21**:295–7.
10. Herrmann K, Niedobitek G. Lack of evidence for an association of Epstein–Barr virus infection with breast carcinoma. *Breast Cancer Res* 2003;**5**:13–7.
11. Brink AA, van den Brule AJ, van Diest P, Meijer CJ. Detection of Epstein–Barr virus in invasive breast cancers. *J Natl Cancer Inst* 2000;**92**:655–6.
12. Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM. No significant association of Epstein–Barr virus infection with invasive breast carcinoma. *Am J Pathol* 2001;**159**:571–8.
13. Labrecque LG, Barnes DM, Fentiman IS, Griffin BE. Epstein–Barr virus in epithelial cell tumors: a breast cancer study. *Cancer Res* 1995;**55**:39–45.
14. Bonnet M, Guinebretiere JM, Kremmer E, et al. Detection of Epstein–Barr virus in invasive breast cancers. *J Natl Cancer Inst* 1999;**91**:1376–81.
15. Fawzy S, Sallam M, Awad NM. Detection of Epstein–Barr virus in breast carcinoma in Egyptian women. *Clin Biochem* 2008;**41**:486–92.

16. Murray PG. Epstein–Barr virus in breast cancer: artefact or aetiological agent? *J Pathol* 2006;**209**:427–9.

17. Andersson TM, Johansson AL, Hsieh CC, Cnattingius S, Lambe M. Increasing incidence of pregnancy-associated breast cancer in Sweden. *Obstet Gynecol* 2009;**114**:568–72.

18. Middleton LP, Amin M, Gwyn K, Theriault R, Sahin A. Breast carcinoma in pregnant women: assessment of clinicopathologic and immunohistochemical features. *Cancer* 2003;**98**:1055–60.

19. Gentilini O, Masullo M, Rotmensz N, et al. Breast cancer diagnosed during pregnancy and lactation: biological features and treatment options. *Eur J Surg Oncol* 2005;**31**:232–6.

20. Haeri S, Baker AM, Boggess KA. Prevalence of Epstein–Barr virus reactivation in pregnancy. *Am J Perinatol* 2010. doi: 10.1055/s-0030-1253098.

21. Avgil M, Ornoy A. Herpes simplex virus and Epstein–Barr virus infections in pregnancy: consequences of neonatal or intrauterine infection. *Reprod Toxicol* 2006;**2**:436–45.

22. Lemire J. 1,25-Dihydroxyvitamin D3 – a hormone with immunomodulatory properties. *Z Rheumatol* 2000;**59**(Suppl. 1): 24–27.

23. Holmøy T. Vitamin D status modulates the immune response to Epstein Barr virus: synergistic effect of risk factors in multiple sclerosis. *Med Hypotheses* 2008;**70**:66–9.

24. Agborsangaya CB, Surcel HM, Toriola AT, et al. Serum 25-hydroxyvitamin D at pregnancy and risk of breast cancer in a prospective study. *Eur J Cancer* 2010;**4**:467–70.

25. Koskela P, Anttila T, Bjørge T, et al. Chlamydia trachomatis infection as a risk factor for invasive cervical cancer. *Int J Cancer* 2000;**85**:35–9.

26. Teppo L, Pukkala E, Lehtonen M. Data quality and quality control of a population-based cancer registry. Experience in Finland. *Acta Oncol* 1994;**33**:365–9.

27. Tedeschi R, Foong YT, Cheng HM, et al. The disease associations of the antibody response against the Epstein–Barr virus transactivator protein ZEBRA can be separated into different epitopes. *J Gen Virol* 1995;**76**:1393–400.

28. Tedeschi R, Bloigu A, Ogmundsdottir HM, et al. Activation of maternal Epstein–Barr virus infection and risk of acute leukemia in the offspring. *Am J Epidemiol* 2007;**165**:134–7.

29. Hollick MF. Vitamin D deficiency. *N Engl J Med* 2007;**357**:266–81.

30. Lehtinen M, Koskela P, Ogmundsdottir HM, et al. Maternal herpesvirus infections and risk of acute lymphoblastic leukemia in the offspring. *Am J Epidemiol* 2003;**158**:207–13.

31. Kazemi A, Sharifi F, Jafari N, Mousavinasab N. High prevalence of vitamin D deficiency among pregnant women and their newborns in an Iranian population. *J Womens Health* 2009;**18**:835–9.

32. Yu CK, Sykes L, Sethi M, Teoh TG, Robinson S. Vitamin D deficiency and supplementation during pregnancy. *Clin Endocrinol* 2009;**70**:685–9.