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Genetic Susceptibility, Lifestyle, Ovarian Hormones, and Mammographic Density - key factors in breast cancer development

The Norwegian Energy Balance and Breast Cancer Aspects-I Study

Anita Iversen

A dissertation for the degree of Philosophiae Doctor

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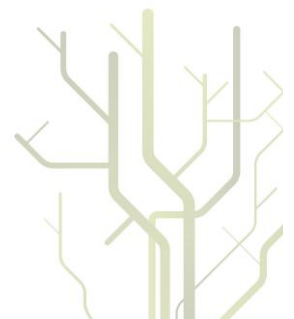

Genetic Susceptibility, Lifestyle, Ovarian Hormones, and Mammographic Density - key factors in breast cancer development

The Norwegian Energy Balance and Breast Cancer Aspects (EBBA)-I Study

"Cancer women" by Sarah Dee

Anita Iversen

Department of Community Medicine

Faculty of Health Sciences, University of Tromsø

Tromsø, Norway
2012

"Women have a unique anatomy and biology.
Women experience other symptoms than men.
Women talk differently about the disease.
Women have less power and influence in the community and in health care in particular.
Women have some other diseases than men."

NOU 1999: 13, Women's health in Norway
Ministry of health and care services, Norway

Preface

From laboratory to epidemiological research

My academic career started as a biomedical laboratory scientist in the 1990s in a laboratory surrounded by mice, cell cultures and continuous upgrades in high-technology analytical equipment. I was studying the puzzle of the immune response when I started my postgraduate work with a main aim to isolate human autoantibodies for use in research, future cancer diagnostics and immunotherapy. This could definitely have been an exciting path to walk. However, after completing the master degree, engagement for health care education on different levels in the organization was in my focus when curiosity and interests led me to a project with a different perspective.

I started my PhD work using data from the cross sectional Energy Balance and Breast cancer Aspects (EBBA)-I Study in 2008 at the Department of Community Medicine, University of Tromsø. The EBBA-I Study is characterized by its broad international collaboration between USA (Peter T. Ellison), Poland (Grazyna Jasienska) and Norway [Inger Thune- Principal Investigator (PI)]. Thune started planning the study already in 1997, with study inclusion in 2000-2002. A parallel study of the EBBA-I Study was conducted at the Jagiellonian University, Krakow, Poland, and all hormonal analyses in saliva were done at the Reproductive Ecology Laboratory led by Peter T. Ellison, Harvard University, Cambridge, MA, USA. A one year stay with Anne McTiernan and her research team at Fred Hutchinson Cancer Research Center in Seattle, WA, USA, was planned in my research project. During the work with the EBBA-I Study and this thesis, collaboration with my supervisors, colleagues at the Department of Community Medicine and an international team of scientists within breast cancer research allowed me to learn about epidemiological breast cancer research with close links to biological mechanisms.

Acknowledgements

There is so many to be grateful to, people that have inspired me and contributed to my work in many different ways. I do hope I haven't forgotten anyone. **Thank you**:

The EBBA-I Study participants: for all the effort you went through by collecting daily saliva, writing diaries and for answering all the questions you were asked.

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The EBBA-I principal investigator- and my co-supervisor Inger Thune: for your enthusiasm, for sharing interesting perspectives through clinical points of views, your creativity and outstanding design of the EBBA-I Study, and for giving me the opportunity to work with such great data.

My co-supervisor Tom Wilsgaard: for always making me feel welcome in your office. I have learned a lot by discussing bio-statistical issues with you, and I highly appreciate your patience and great skills in mentoring.

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My fellow PhD students at the research school EPINOR, ISM, UiT: for all the questions and knowledge you brought into my learning environment, all the fun we had over the last four years and the encouragement that kept me moving forward. I hope we manage to stay in touch.

My colleagues at ISM: for being true believers, hard workers and an absolutely multitudinous group of people. For all your great inputs and all questions you asked for me to reflect upon. I will miss you all.

My colleagues at the Faculty of Health Sciences: for your interest in me and my work, your support and everlasting friendship, and Arnfinn Andersen for being optimistic and supportive of my decision in 2007, I will not forget your thoughtful and vice leadership.

To Pam, David, Bonnie and Steve, Andy, and Janice: for all the support and interest in my work and wellbeing. Because of you, I have second families where I feel welcome at all times. There will always be a next visit to look forward to.

Frode: for being my best friend and love of my life. Words can hardly explain my gratitude to you. All the time you spent listening to my work - you truly must have been bored sometimes?! I admire you for your thoughtfulness and lack of selfishness, and I will always remember your endless support.

My dear children Joakim, Marius and Kristine: for inspiring me by being yourself and giving me an opportunity to spend energy on you, too. You are simply the best!

Dear mom, dad, Gunn and Sverre: for filling my heart with love and tenderness and my freezer with fish, berries and all kinds of healthy homemade food.

Berit, Hege, Tor and Stein Erik and their families: for thoughtful attention and great questions during my work. I definitely love you all.

Lutfisklaget L.O.P and The gourmet club V.E.F.E.M: A PhD-project can be a very lonely ride, so thank you for dragging me out there and adding important texture to my life!

Anita

Tromsø, November 2012

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Summary

Breast cancer is the most frequent cancer among women worldwide. Over the last century, the prevalence of overnutrition, overweight and obesity has increased, age at menarche has dropped and age at birth of first child has risen among women in Western countries. These trends may be linked to exposure to ovarian hormones that, in turn, may affect a woman's lifetime risk of getting breast cancer.

The Norwegian Energy Balance and Breast cancer Aspects (EBBA)-I Study includes ovarian hormone data from complete menstrual cycles, blood samples, mammograms, clinical measurements, and lifestyle information from 204 healthy premenopausal women aged 25-35 years. The study is designed to explore biological mechanisms linking energy balance and hormonal exposures with breast cancer risk, and to identify new risk patterns of importance for prediction, prevention and treatment of breast cancer. The aim of this thesis was to study levels of endogenous estradiol and progesterone in relation to genetic susceptibility, metabolic and reproductive risk factors, and mammographic density, among healthy premenopausal women using data from the EBBA-I Study.

The results from the present analyses show that healthy premenopausal women with the *CYP17* rs2486758 minor allele in combination with higher levels of metabolic risk factors had higher levels of estradiol across the menstrual cycle. Larger waist circumference and longer duration of past use of oral contraceptives were associated with higher levels of estradiol across the menstrual cycle among nulliparous women. Higher levels of estradiol and progesterone were strongly associated with higher mammographic density, particularly among nulliparous women. From our findings, we hypothesize that interventions to lower hormonal levels in premenopausal women could decrease mammographic density and improve the sensitivity of mammographic screening, both of which could improve breast cancer prevention. These interventions may be particularly important in premenopausal women before first full-term pregnancy and in a subgroup of women with a specific genetic marker.

Sammendrag

Brystkreft er den hyppigste kreftformen blant kvinner på verdensbasis. I løpet av det siste århundret har forekomsten av overernæring, overvekt og fedme økt, samtidig som alder ved menarke har falt og alder ved første fødsel har steget blant kvinner i vestlige land. Disse trendene kan påvirke nivå av kvinnelige kjønnshormoner og dermed kvinners brystkreftrisiko gjennom livet.

Den norske EBBA-I studien (Energy Balance and Breast cancer Aspects) inkluderer daglige målinger av kvinnelige kjønnshormoner gjennom menstruasjonssyklus, blodprøver, mammogram, kliniske undersøkelser, og informasjon om livsstil fra 204 friske premenopausale kvinner i alderen 25-35 år. Studien er designet for å utforske biologiske mekanismer som knytter energibalanse og kvinnelige kjønnshormoner til brystkreftrisiko, og frembringe ny kunnskap for bedre forebygging og behandling av brystkreft. Målsettingen med denne avhandlingen var å studere nivå av endogent østradiol og progesteron i relasjon til genetisk sårbarhet, reproduksjon, metabolske risikofaktorer og mammografisk tetthet hos kvinner i EBBA-I studien.

Resultatene i denne avhandlingen viser at friske premenopausale kvinner med en enkeltbasevariant i *CYP17* (rs2486758) og høyest nivå av metabolske risikofaktorer hadde høyere nivå av østradiol gjennom menstruasjonssyklus. Større livvidde og lengre varighet av tidligere p-pillebruk var assosiert med høyere nivå av østradiol gjennom menstruasjonssyklus hos kvinner som ikke hadde født barn. Høyere nivå av østradiol og progesteron var sterkt assosiert med høyere mammografitetthet, særlig blant kvinner som ikke hadde født barn. På bakgrunn av våre funn er hypotesen at intervensjoner som senker nivå av endogene kvinnelige kjønnshormoner, kan redusere mammografitetthet og forbedre sensitiviteten ved mammografi hos unge kvinner. Slike intervensjoner kan ha betydning for forebygging av brystkreft og være særlig viktig hos kvinner før første fullgåtte svangerskap og for unge kvinner med en spesifikk genetisk markør.

List of papers

This thesis is based on the following three papers, which will be referred to in the text by their Roman numeral:

Paper I

Iversen A, Thune I, McTiernan A, Makar KW, Wilsgaard T, Ellison PT, Jasienska G, Flote V, Poole EM, Furberg A-S. Genetic polymorphism *CYP17* rs2486758 and metabolic risk factors predict daily salivary 17β-estradiol concentration in healthy premenopausal Norwegian women. The EBBA-I Study. Journal of Clinical Endocrinology and Metabolism 97:E852-E857, 2012

Paper II

Iversen A, Thune I, McTiernan A, Emaus A, Finstad SE, Flote V, Wilsgaard T, Lipson S, Ellison PT, Jasienska G, Furberg A-S. Ovarian hormones and reproductive risk factors for breast cancer in premenopausal women: the Norwegian EBBA-I Study. Human Reproduction 26:1519-1529, 2011

Paper III

Iversen A, Furberg A-S, McTiernan A, Finstad SE, McTiernan A, Flote V, Alhaidari G, Ursin G, Wilsgaard T, Ellison PT, Jasienska G, Thune I. Associations of daily 17β-estradiol and progesterone with mammographic density in premenopausal women. The Norwegian EBBA-I Study. *Submitted*

1 Introduction

1.1 Background

The endogenous ovarian hormones estradiol and progesterone play an important role both in the normal growth and development of the breast and in breast cancer development. Breast cancer is the most frequent cancer among women; in 2010, the estimated number of new cases among women worldwide was 1.44 million (Globocan) and 2839 new cases were diagnosed among women in Norway (Cancer Registry of Norway). There is at least a 10-fold variation in breast cancer incidence rates worldwide (Globocan, Ferlay et al. 2010), largely as a consequence of a range of socio-economically correlated differences in the population prevalence of nutritional, reproductive, metabolic and hormonal factors, and differences in genetic background. It is generally thought that the link between energy balance and breast cancer risk is partly mediated by ovarian sensitivity to nutritional status during susceptible life stages (Jasienska and Thune 2001, Robsahm and Tretli 2002, AICR 2007, Bukowski et al. 2012, Weedon-Fekjær et al. 2012). Both ovarian function and breast cancer risk may be modified by lifestyle (Jasienska et al. 2000, IARC 2002, AICR 2007). Thus, a woman's menstrual cycle profile in premenopausal years may represent a valuable biomarker for later breast cancer risk.

Over recent years, many countries and subpopulations worldwide have experienced excess weight, earlier onset of menarche and postponement of pregnancies, with urban-rural differences in trends. These trends may be linked to variation in exposure to ovarian hormones that could affect the lifetime risk of getting breast cancer (Jasienska and Thune 2001, Robsahm and Tretli 2002, Opdahl et al. 2011, Fredslund and Bonefeld- Jørgensen 2012). The impact of ovarian hormones on breast carcinogenesis may be particularly strong during the time period before any full-term pregnancy, as the immature breast tissue is more susceptible to exposures (Trichopoulos et al. 2005, Opdahl et al. 2011, Johnson et al. 2012).

Breast cancer is a heterogeneous disease in which risk is manifested through both genes and environmental factors (Giarelli et al. 2005, AICR 2007, Nelson et al. 2012). Studies

including molecular determinants of ovarian hormone synthesis and metabolism may reveal important clues about genetic susceptibility and gene-lifestyle interactions in relation to breast cancer risk.

Mammographic density is the strongest risk factor for breast cancer apart from age and gender (Boyd et al. 2005, Palomares et al. 2006, McCormack et al. 2006). Knowledge is limited regarding the time interval necessary for the transition from a normal to a neoplastic breast epithelial cell, and for the promotion and progression of breast cancer. Thus, predictors of mammographic density in young premenopausal women may provide knowledge about susceptibility to breast cancer of particular importance in clinical practice.

The Energy Balance and Breast cancer Aspects (EBBA)-I Study aims to provide new insight into biological mechanisms linking energy balance and hormonal exposures with breast cancer risk, and to identify new risk patterns of importance for prediction, prevention and treatment of breast cancer.

On this background, I chose to focus the present thesis on studying levels of endogenous 17β-estradiol and progesterone in relation to genetic susceptibility, metabolic and reproductive risk factors, and mammographic density in premenopausal women using data from the EBBA-I Study conducted in North-Norway.

1.2 Estrogen and progesterone

The endogenous ovarian hormones estrogen and progesterone are steroid molecules derived from cholesterol (Figure 1-2) and are produced in the ovaries, placenta, adrenal cortex and adipose tissue (Stanczyk and Bretsky 2003, Ghayee and Auchus 2007).

Endogenous estrogen exists as three distinct molecules in circulation with different predominance related to menopausal phase. Estradiol (17β-estradiol) is highly potent and predominant in premenopausal years, while estrone is mainly derived from adipose tissue, less potent and predominant in postmenopausal women. Estriol is synthesized in placenta during pregnancy and is considered the weakest estrogen (Zhu and Conney 1998, Clemons

and Gross 2001). Endogenous progesterone is the predominant ovarian hormone during pregnancy.

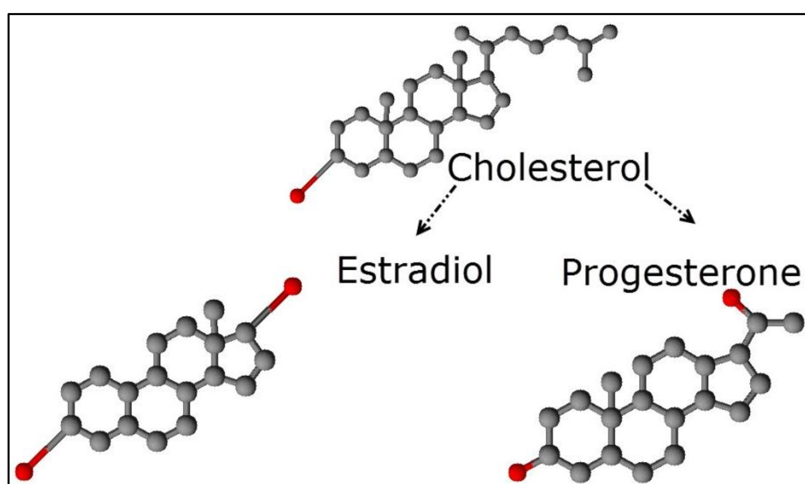


Figure 1. Three-dimensional structure of cholesterol, estradiol and progesterone. Designed by Homayoun Amirnejad, 2012.

In women of reproductive age, biologically active estrogen is primarily secreted from the ovaries as estradiol. In addition, androstenedione secreted in equal amounts from the adrenal glands and ovaries in premenopausal women acts as a precursor and contributes to the circulating levels of estrone through conversion in extraglandular tissues. Estrone may be further converted to estradiol depending on the presence of an enzyme-complex including aromatase, which is the key enzyme responsible for the aromatization of androgens to estrogens. The aromatase enzyme is found in many tissues including the ovaries, placenta, endometrium, as well as in adipose tissue, bone, skin, and in normal as well as cancerous breast tissue.

In circulation, most estradiol remains strongly bound to sex-hormone binding globulin (SHBG); some is weakly bound to albumin, and only a small portion remains free (1-2%) and unbound to carrier proteins (Chiappin et al. 2007). Unbound estradiol molecules can pass through the capillary wall, the basement membrane and the membrane of the salivary glandular epithelial cells to be secreted in saliva (Dunn et al. 1981, Lipson and Ellison 1989). Estradiol is metabolized and excreted primarily through the kidney, but also partially through the intestine, appearing in urine and feces, respectively (Stanczyk and Bretsky 2003). Circulating concentrations of ovarian hormones among premenopausal women vary

throughout the menstrual cycle with dominating levels of estradiol in the proliferative or follicular phase, with a peak on the day before ovulation. The early follicular and late luteal phases of the menstrual cycle are characterized by low levels of estradiol. Specific follicular and luteal estradiol indices, including maximum peak concentration were calculated and used in the statistical analyses for Papers I-III in this thesis (Figure 13).

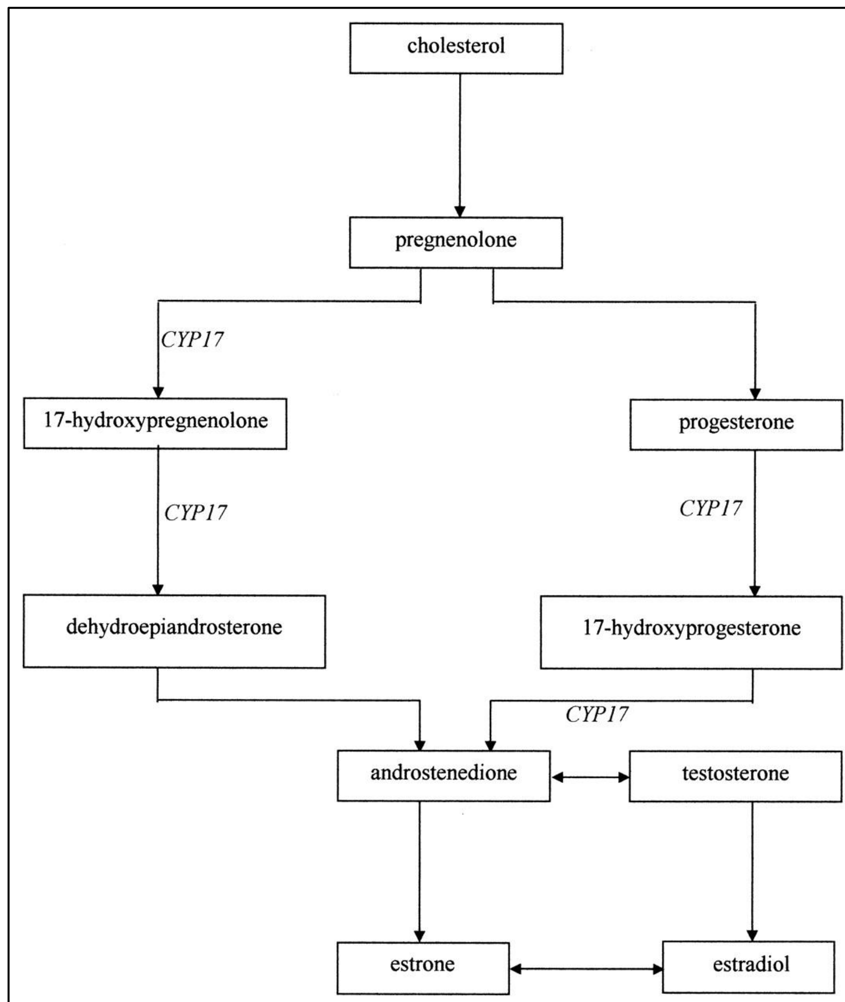


Figure 2. The steroidogenic pathway in the ovary for estrogen and progesterone synthesis. (Reprinted from Sharp L et al. Am J Epidemiol 160:729-740, 2004, with permission from Oxford University Press).

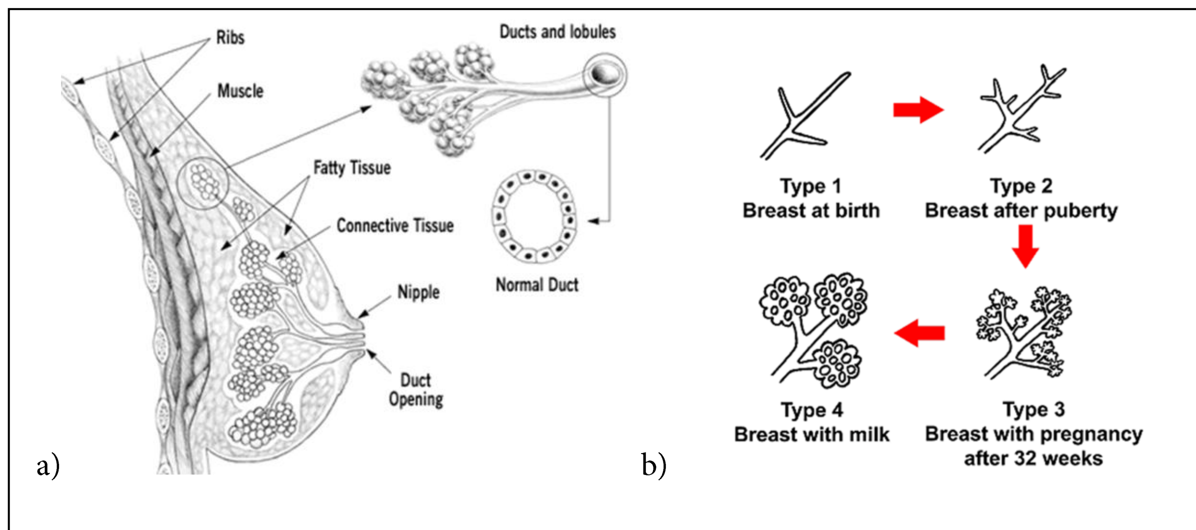
Progesterone is released from the corpus luteum after ovulation. In circulation, progesterone is bound to corticosteroid-binding globulin (CBG) (Misao et al. 1999) and, like estradiol, progesterone is also weakly bound to albumin resulting in low levels of free progesterone. Unbound progesterone has the same ability as estradiol to pass from circulation to saliva, and become metabolized and excreted, appearing in the urine and the feces (Stanczyk and Bretsky 2003). During the secretory or luteal phase of the menstrual

cycle, progesterone serves as the major ovarian hormone, and different luteal progesterone indices were used in the analyses presented in Papers II-III (Figure 13). In the follicular phase of the menstrual cycle, the amount of progesterone is negligible.

Specific receptors, specifically estrogen receptor (ER) and progesterone receptor (PR), are mainly expressed in the cytoplasm or the membrane of the nucleus of epithelial cells in the breast, as well as in the endometrium and brain tissue in women. Low-affinity bound and free ovarian hormones exert biologic effects by binding to their specific receptors (Tanos et al. 2012). The hormone-receptor complex enters the nucleus, binds to DNA, and induces or represses transcription of target genes. Ovarian hormones are suggested to play a role in the regulation of the ER and PR expression (Yang et al. 2010) and the percentage of cells in the breast tissue expressing ER and PR varies throughout stages of breast development (Russo and Russo 2004a). Both the ER and the PR have two known isoforms, α and β, which regulate different genes (Kumar et al. 1987, Giangrande et al 2000, Leitman et al. 2010).

1.3 Normal breast development

The mammary glands start to develop during fetal life when the embryo is less than 5 mm. At birth, the breast tissue is characterized by primitive structures consisting of luminal epithelial cells and myoepithelial cells (DeVita et al. 2011, Stingl 2011), which form the ducts that later will be used for milk transportation to the nipple (Figure 3a). There are considerable changes in female breast cells and tissue throughout life that also affect susceptibility to potential carcinogenetic factors. During childhood the breast cells and tissue are generally considered to be unaltered. In puberty, hormonal changes and growth factors induce the development of the connective tissue surrounding the mammary ducts. The amount of adipose tissue increases, and at menarche, estradiol promotes further growth of the ductal system of the breast, while progesterone is responsible for the promotion of alveolar development of the breast glands. The multiple terminal ducts ending with alveolar buds form terminal ductal lobuloalveolar units named type 1 lobules (Figure 3b) (Russo and Russo 2004a, DeVita et al. 2011).



Figur 3. Illustration of a) Normal breast anatomy and b) Types of breast lobules. (Reprinted with permission from www.my-breast-cancer-guide.com).

The lobules formation continues until about age 35, and involves further differentiation into type 2 and 3 lobules. The final development and maturation of the mammary glands take places after the first full-term pregnancy with the proliferation of the alveolar buds into ductules (acini secreting units) and type 4 lobules formation. Breast tissue in nulliparous women contains predominately types 1 and 2 lobules, whereas in parous women, most of the mammary glands have differentiated to type 3 and 4 lobules (Russo and Russo 2004a, DeVita et al. 2011). At menopause, both nulliparous and parous women experience a cessation of ovarian hormones and a regression of the breast tissue, resulting in an increased number of type 1 lobules, and a decline in the number of other lobule types.

1.4 Breast cancer

1.4.1 Occurrence

Breast cancer has for decades been the most frequent cancer among women in Norway, and 2839 new invasive breast cancer cases were diagnosed in 2010. Furthermore, for women aged less than 65 years, breast cancer is the most important cause of lost life years (Cancer Registry of Norway). Breast cancer ranks as the fifth cause of death among women worldwide, with a total of 450 000 deaths in 2008 (Globocan). In Norway, 673 women died of breast cancer in 2010 (Norwegian Institute of Public health). Estimations based on data

from the 5-year time period 2006-2010 indicate that Norwegian women have a 7.9 % (1:13) average lifetime risk of incident breast cancer (Cancer Registry of Norway). In comparison, the lifetime risk of getting breast cancer is approximately 1:8 and 1:40 among women in USA and Asia, respectively (Howlader et al. 2012, Globocan).

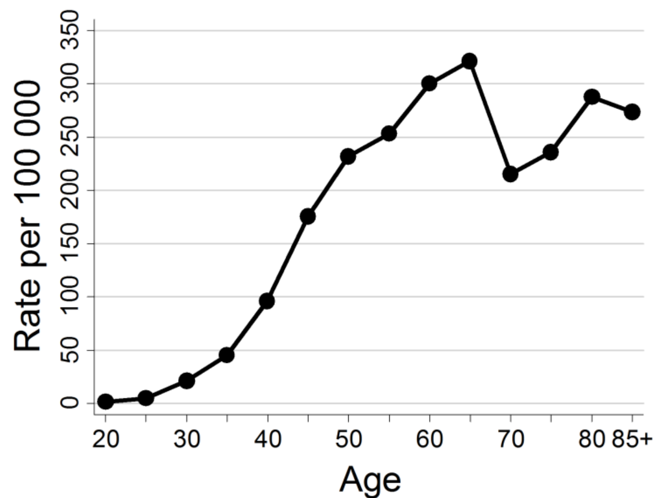


Figure 4. Age-specific breast cancer incidence (rates pr 100 000) in Norwegian women , 2005-2009. (Engholm et al. NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, http://www.ancr.nu, accessed on 08/09/2012).

The age-specific incidence curve of breast cancer shows that invasive breast cancer is diagnosed in young women (below 20 years). During premenopausal years a steep increase in incidence is observed, with a small break in the curve around menopause (age 50) (Figure 4), termed the Clemmensen's Hook. It is hypothesized that these changes in breast cancer incidence reflect the impact of varying ovarian hormone exposure throughout life.

The increasing trend in breast cancer incidence during the last 50 years in Norway, leveled off in 2005, and has continued to decline (Figure 5). This decline is partly explained by a reduction in the use of hormone therapy among postmenopausal women (Beral et al. 2003, Wedon Fekjær et al 2012).

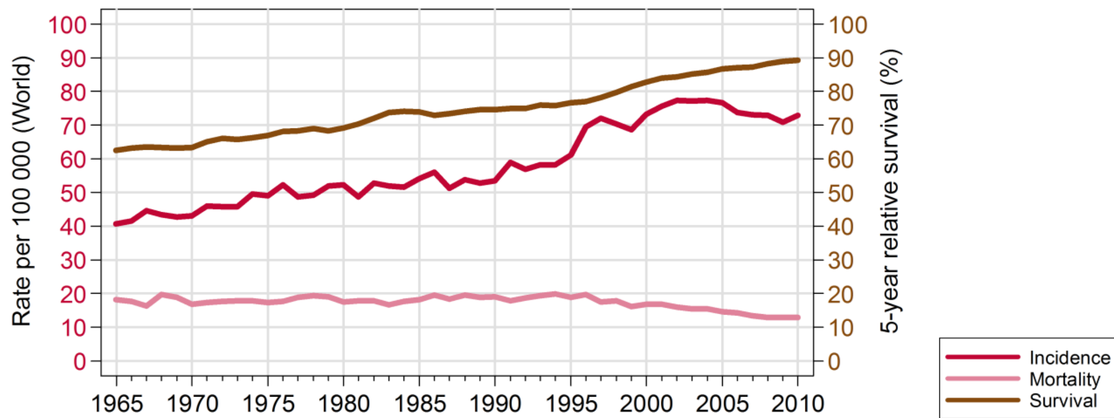


Figure 5. Trends in age-standardized (world) incidence and mortality rates and 5-year relative survival proportions for breast cancer among women in Norway. (Reprinted with permission from: Cancer Registry of Norway. Cancer in Norway 2010 – Cancer incidence, mortality, survival and prevalence in Norway. Oslo: Cancer Registry of Norway, 2012).

It has also been suggested that mammographic screening has had a longstanding influence on breast cancer incidence in Norway (Figure 6) (Weedon-Fekjær et al. 2012), with variations in significance across age-groups (Hofvind et al. 2012). Similar trends in breast cancer incidence and possible explanations have likewise been reported by others in Europe (Pollán et al. 2009, Crocetti et al. 2010), the USA (Hausauer et al. 2009, Marshall 2010), and Australia (Canfell et al. 2008). There has been a steady increase in five-year relative survival of breast cancer in Norway, including all tumor types and age-groups combined, from 66.5 % in 1971-1975 to a current survival rate of 88.7 % in 2006-2010, and from 1996 the breast cancer mortality started to decline (Figure 5). Therefore, in 2010, 37079 breast cancer survivors were still alive in Norway. The drop in mortality and the increased survival rate may partly be explained by greater knowledge related to prevention and treatment of breast cancer. Advances in primary prevention include the detection of premalignant disease, resulting in the detection of smaller and less advanced disease. Improved treatment modalities in surgery and oncology compared to just a decade ago include tailored treatment modalities related to surgery, endocrine therapy, chemotherapy, treatment with antibodies and radiation. Therefore, Norway as well as other countries have experienced an increase in prevalent cases of breast cancer among women of all ages, including women

with metastatic disease alive 5-10 years after diagnosis. As a result, breast cancer is now considered more or less a chronic disease.

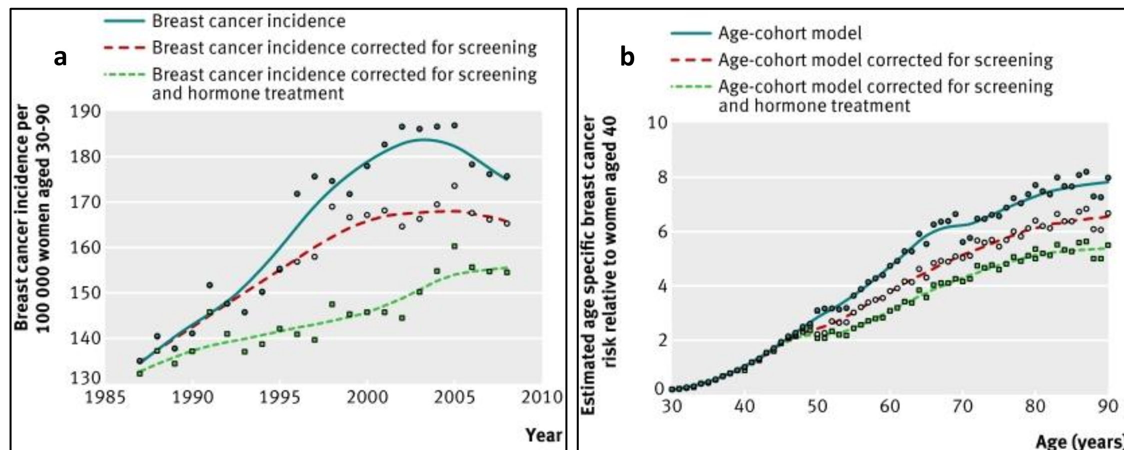


Figure 6. a) Breast cancer incidences and b) estimated age-specific risk among women in Norway. (Reprinted from Weedon-Fekjær et al BMJ 2012, 344:e299, with permission from http://creativecommons.org/licenses/by/2.0).

Global trends regarding breast cancer incidence, mortality, survival and prevalence show variation between countries and between rural and urban areas. These differences in invasive breast cancer incidence may reflect variation in life expectancy, mammographic screening programs, as well as genetic susceptibility (Guo et al. 2012). Moreover, these observations support the notion that nutritional, reproductive and hormonal factors will continue to influence the burden of breast cancer toward 2030 (Bray et al. 2012).

1.4.2 Breast cancer development

Breast cancer development results from uncontrolled growth of mammary gland cells (Figure 7), localized to the ducts in about 75 % of cases (ductal breast carcinoma), or the lobules in 5 to 15 % of cases (lobular breast carcinoma). Breast cancer has a heterogeneous etiology. Not all subtypes of breast cancer are caused by the same underlying biology (Polyak 2007), or have the same stem cell origin (Kai et al. 2010), suggesting that different risk factors may exist for different breast cancer subtypes. Thus, breast cancer therapy and prognosis are determined by patient characteristics [e.g. age, family history of cancer, prior predisposing factors (i.e. fibroadenomatosis, previous cancer), reproductive history, and

menopausal status], tumor size and lymph node status (TNM-classification), as well as other tumor characteristics [histological type, proliferation marker (Ki67), other tumor markers (ER/PR status), human epidermal growth factor receptor (HER2), breast cancer gene mutations (e.g. *BRCA1* and *BRCA2*), tumor protein 53 (encoded by *TP*53), vascular invasion, and necrosis]. In Norway, national guidelines for adjuvant and palliative breast cancer treatment are evaluated and updated at least twice annually by the Norwegian Breast Cancer Group according to national and international reports (NBCG 2012).

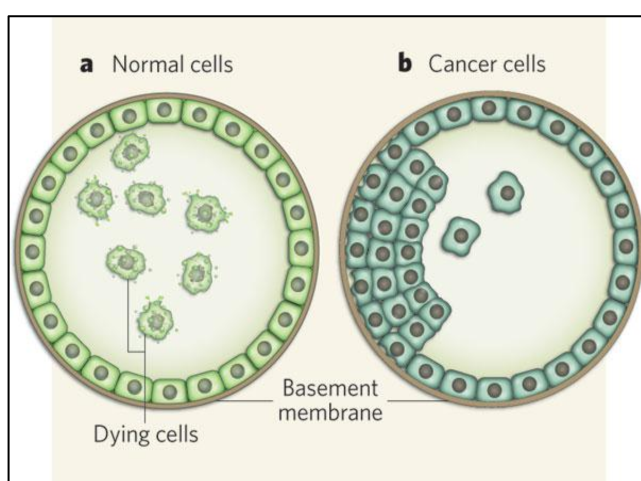


Figure 7. Illustration of a) Normal breast epithelial cells that are damaged beyond repair and eliminated by apoptosis, and b) Cancer cells that avoid apoptosis, survive and continue to multiply in an unregulated manner. (Reprinted from Gottlieb E, Nature 461: 44-45, 2009, with permission from Nature Publishing Group).

Around 65-95 % of all breast cancers have recently been observed to be ER and/or PR positive tumors (Menashe et al. 2009, Steindorf et al. 2012, NBCG 2012). The large variation in receptor status may partly be explained by variation in ethnicity, age, menopausal status and lifestyle factors of importance for breast cancer development between study populations.

Estrogen is a potent breast mitogen (Henderson et al. 1982, Preston-Martin et al. 1990, Pike et al. 2007), and several mechanisms have been proposed to explain its role in breast cancer development. One of the proposed mechanisms suggests that ER signaling promotes cell proliferation, decreases apoptosis (cell death) and increases opportunities for errors in DNA (deoxyribonucleic acid), leading to carcinogenesis (Yager and Liehr 1996, Clemons and Gross 2001, Russo and Russo 2004b). Another suggested mechanism is that reactive oxygen species (ROS) and/or estrogen metabolites generated during a cytochrome P450-

mediated estrogen metabolism, react covalently with DNA causing direct genotoxic effects by increasing mutation rates (Yue et al. 2003, Yager and Davidson 2006). A third mechanism has also been suggested and involves regulation of enzyme activities or transcription factors involved in redox signaling by estrogen induced ROS (Chang 2011).

Levels of circulating endogenous estrogen in postmenopausal women have been associated with a two to three fold increased breast cancer risk (Cauley et al. 1999, Key and Allen 2002, Travis and Key 2003, Eliassen et al. 2006a), independently of tumor ER and PR status (Baglietto et al. 2010). Among premenopausal women, the relationship between ovarian hormones and risk of breast cancer is complicated due to the fluctuating ovarian hormonal levels throughout the menstrual cycle (Hankinson and Eliassen 2010). However, a recent meta-analysis of nine pooled prospective studies found that a doubling of circulating estradiol levels in premenopausal women was associated with 10 % higher risk of breast cancer (Walker et al. 2011), in contrast to what had been previously reported by others (Kaaks et al. 2005, Eliassen et al. 2006b).

In contrast to estrogen, the role of progesterone in breast cancer development is not clear (Lange and Yee 2008). However, the PR isoform ratio is suggested to influence breast cancer progression (Khan et al. 2012) and it has been hypothesized that high cell proliferation of breast epithelium in the luteal phase compared to the follicular phase of the menstrual cycle, may indicate a mitotic effect of progesterone (Ferguson and Anderson 1981, Key and Pike 1988, Söderqvist et al. 1997). Furthermore, experiments in rodents have shown that proliferation in normal glands and mammary carcinogenesis in rats requires prolonged exposure to both estrogen and progesterone (Blank et al. 2008, Kariagina et al. 2010), suggesting a synergy between the ovarian hormones. This hypothesis was partly supported in the Women's Health Initiative randomized controlled trial (Rossouw et al. 2002, Anderson et al. 2004) and by others (Beral et al. 2003), where postmenopausal women taking progestin in combination with estrogen experienced a higher breast cancer risk relative to those taking estrogen alone. Furthermore, hormone therapy among

postmenopausal women has been associated with up to two-fold increased postmenopausal breast cancer risk (Colditz et al. 1995, Collaborative group on Hormonal factors in Breast cancer 1997, Beral et al. 2003). So far, studies among both premenopausal (Eliassen et al. 2006b) and postmenopausal women (Missmer et al. 2004) have reported no association between serum progesterone and risk of breast cancer.

Oral contraceptives are sources of exogenous estrogen and progesterone in premenopausal women and longer duration of oral contraceptive use has been associated with an increased breast cancer risk among premenopausal women (Zhu et al. 2012). The association between use of oral contraceptives and breast cancer risk in postmenopausal women is less clear. However, greater risk of breast cancer in current users of hormone therapy with a history of oral contraceptive use has been observed in Norway (Lund et al. 2007).

1.5 Breast cancer risk factors

Breast cancer risk factors, other than being a woman and aging, include: known genetic susceptibility (*BRCA1*, *BRCA2*, *PTEN*, *TP53*), family history of breast cancer, contralateral breast cancer, benign tumors (e.g. fibroadenomatosis), excessive radiation, lifestyle factors (e.g. physical inactivity, alcohol, obesity), high levels of various hormones (e.g. hormone therapy), and high mammographic density (McCormack et al. 2006, Gray et al. 2009). Most of the established risk factors reflect the “cumulative” dose of estrogens that the breast epithelium is exposed to throughout life (Henderson et al. 1982, Clemons and Gross 2001, Bernstein 2002, AICR 2007). Therefore, in the following sections, evidence for most of the risk factors included in Papers I-III of this thesis are described in relation to breast cancer risk and cumulative and/or circulating levels of endogenous ovarian hormones.

1.5.1 Genetic susceptibility-the single nucleotide polymorphism (SNP)

Breast cancers associated with inheritance are mostly caused by the mutation or deletion of genes involved in critical cell functions such as DNA repair, proliferation, cell cycle control, and apoptosis (Nasca and Pastides 2008). Rare mutations in the tumor-suppressor genes, *BRCA1* and *BRCA2* confer up to 80 % lifetime risk of breast cancer among mutation

carriers, but lifestyle exposures may trigger or delay disease onset (King et al. 2003). Germline mutations in the tumor-suppressor genes *TP53* and *PTEN* with low prevalence in the general population, lead to high breast cancer risk (30-90 %) (Santen 2008). However, only 5-10 % of breast cancer occurrence is accounted for by these high risk mutations (Newman et al. 1998, Ellisen and Haber 1998, Balmain et al. 2003, Fanale et al. 2012). This suggests that common genetic variants associated with low increases in risk may account for most breast cancer cases (Mcinerney et al. 2009). Interestingly, about 12 % of the population has a lifetime risk of at least 10 % for developing breast cancer, and 50 % of all breast cancers develop in this subpopulation (Balmain et al. 2003).

The most common sequence variation in the genome, the single nucleotide polymorphism (SNP), is a stable substitution of a single nucleotide, which by definition is observed in at least 1 % of a population (Rothman, 2011) (Figure 8). A haplotype may be defined as a group of inherited SNPs that occur in predictable patterns within sections of DNA. Combinations of SNPs, either as haplotypes or between distant genes, may coordinately contribute to the etiology of a specific cancer (Chanock 2001, Kotnis et al. 2005) by influencing gene expression (Manolio 2010).

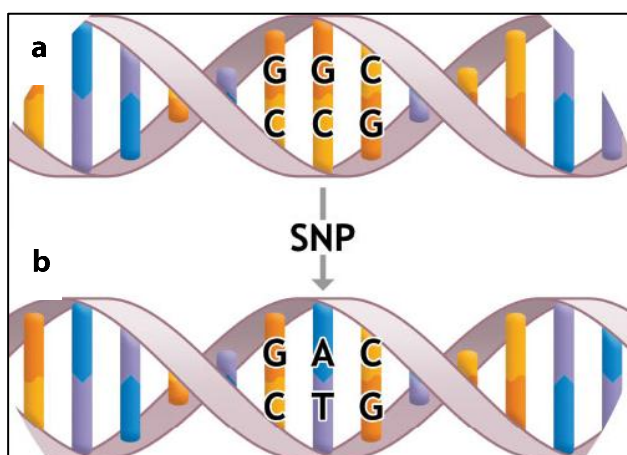


Figure 8. The DNA molecule in a) differs from the DNA molecule in b) at a single nucleotide-pair location [GC in a) versus AT in b)], called a single nucleotide polymorphism (SNP). (Reprinted with permission from http://www.ibbl.lu/. Rights of reproduction are reserved and limited).

Candidate variants in key genes such as functional or regulatory SNPs can provide useful information in understanding biological mechanisms and biologically plausible pathways (Erichsen and Chanock 2004), although advances in technology have made more comprehensive studies possible. Thus, genetic polymorphisms in genes (i.e. *CYP17*, *CYP19,*

HSD17B, CYP1A1, CYP1B1, HSD3B, COMT, GSTM1, GSTM3, GSTP1, GSTT1 and *MnSOD*, *SULT1A1, UGT1A1)* (Mitrunen and Hirvonen 2003) encoding key enzymes in steroid biosynthesis and metabolism may be strong biomarkers for breast cancer susceptibility (Cerne et al. 2011) or variation in mammographic density (Crandall et al. 2009). Even if low penetrance genes only moderately elevate risk of breast cancer, it may be of great value to consider the role of genetic variation in understanding lifestyle factors and potential avenues for intervention (Palmer et al. 2011). Estrogen and progesterone synthesis is regulated by a network of enzymes encoded by different genes (Clemons and Gross 2001). Thus, it is reasonable to hypothesize that genetic variation within *CYP17* in the steroidogenic pathway could influence levels of ovarian hormones alone or in interaction with lifestyle factors of importance for breast cancer development.

CYP17-genotype

Genes encoding cytochrome P450 enzymes, and the enzymes themselves, are designated with the abbreviation CYP. The enzymes encoded by the *CYP17* gene located on chromosome 10 function as key branch points in the steroidogenic pathway (Clemons and Gross 2001, Stanczyk and Bretsky 2003, Ghayee and Auchus 2007, Patel et al. 2010), (Figure 2). The rs743572 in the promoter region of *CYP17* is the most studied SNP on this gene in relation to risk of breast cancer, and systematic reviews have reported conflicting results (Dunning et al. 1998, Feigelson et al. 2002). Also, a more recent meta-analysis among premenopausal and postmenopausal women confirmed the overall null association between the *CYP17* rs743572 and risk of breast cancer (Chen and Pei 2010). Interestingly, the *CYP17* hetero- and homozygote minor allele have been suggested to have an impact on premenopausal breast cancer risk among nulliparous women (Verla- Tebit et al. 2005), and to modify the associations between estrogen-like chemicals (e.g. lignan) and premenopausal breast cancer risk (Piller et al. 2006). In a subgroup analysis, possible correlations between menopausal status, age at menarche, body mass index (BMI) and *CYP17* polymorphism were observed (Chen and Pei 2010). Some previous studies have reported increased levels of salivary and serum estradiol among healthy premenopausal women with the hetero- and

homozygote genotype of the *CYP17* rs743572 minor allele (Feigelson et al. 1998, Small et al. 2005, Jasienska et al. 2006), while others reported inconsistent results for estradiol (Garcia-Closas et al. 2002) as well as for serum progesterone (Feigelson et al. 1998, Garcia-Closas et al. 2002).

The inconsistency in reported findings of associations between *CYP17* genotypes and levels of ovarian hormones may in part be explained by methodological issues with respect to the measurement of ovarian hormone and the challenges with fluctuating hormone levels throughout the menstrual cycle. There is a lack of studies testing whether *CYP17* SNPs other than rs743572 are associated with levels of estradiol and progesterone throughout the entire menstrual cycle among premenopausal women.

1.5.2 Reproductive factors

The time interval from age at menarche to age at first full-term pregnancy may refer to a particularly susceptible period for initiation of breast cancer as the mammary glands and cells are not fully developed (Russo et al.1982, Pike et al. 1983, Hulka and Stark 1995). The trend towards a gradual decline in age at menarche from approximately 17 years in the early 19th century to approximately 13 years by the mid-20th century has been reported in Norway as well as worldwide (Sørensen et al. 2012). This observation parallels a recent trend of postponing pregnancies, with a mean age at first childbirth of 28.4 years in 2011 compared with 22.6 years in 1970 (Statistics Norway, Norwegian Institute of Public Health). These trends translate into a longer 'menarche-to-first birth' interval, which has been associated with a 1.5 fold increased risk of hormonally sensitive types of breast cancer (Li et al. 2008).

The cumulative number of ovulatory cycles in a woman's life has also been related to risk of breast cancer (Pike et al. 1983, Bernstein 1993, den Tonkellar and de Waard 1996, Garland et al. 1998). The underlying mechanisms between these associations are purported to include prolonged exposure of ovarian hormones. However, studies of ovarian hormones

levels across a menstrual cycle in relation to the 'menarche-to-first birth' interval are limited.

During the last century, the mean number of childbirths among Norwegian women has decreased from 4.4 childbirths to 1.88. The largest relative decrease in childbirths has been observed in rural areas compared to cities, and among native born women by comparison with immigrant women in Norway (Statistics Norway, Norwegian Institute of Public Health). Furthermore, when comparing Norwegian women aged 45 in 1935 versus 1965, the total number of nulliparous women has increased from 9.6 % to 12.2 % (Statistics Norway). Parity has a strong protective effect on breast cancer risk (Lund 1990, Kelsey 1993, AICR 2007), and the underlying mechanisms may include differentiation of mammary epithelial cells, reduced numbers of mammary stem cells, altered response to ovarian hormones, reduced levels of circulating hormones (Britt 2007), and breast feeding (Kelsey 1993, Ursin 2005). Furthermore, nulliparity is related to increased breast cancer risk (Van Gils 2000, Newcomb 2011), more aggressive breast cancers (Butt 2009), and increased breast cancer mortality (Lund 1990, AICR 2007), and may reflect the increased susceptibility to carcinogens in the undifferentiated mammary gland cells in these women. Studies of parity in relation to ovarian hormones throughout the menstrual cycle are limited.

1.5.3 Lifestyle factors

Overweight and obesity reflected by either a high BMI or waist circumference are indications of positive energy balance, and are associated with a 30-50 % increased postmenopausal breast cancer risk (Thomas et al. 1997, Huang et al. 1999, IARC 2002, Ballard-Barbash et al 2006, AICR 2007, Key et al. 2003). This may, at least in part, be explained by increased estrogen levels among overweight and obese postmenopausal women due to the conversion of estrogen from androgens in adipose tissue. In contrast, obesity among premenopausal women has been associated with a 30 % lower relative risk of breast cancer (Ballard-Barbash et al 2006, Bjørge et al. 2010, Green et al. 2012), thought to

be explained by more frequent anovulatory cyclers, longer menstrual cycles and lower estradiol exposure among obese young women. Reports on the association between waist circumference and risk of breast cancer in premenopausal women are inconsistent (Hajian-Tilakiand et al. 2011, Harris et al. 2011), and vary by tumor receptor status (Ritte et al. 2012). In premenopausal women, adult BMI has been strongly inversely related to circulating estrogen levels (Tworoger et al. 2006) and progesterone levels (Tworoger et al. 2006, Yeung et al. 2012), while positively associated with calculated levels of free estradiol (Yeung et al. 2012). Furthermore, higher BMI and higher serum low-density / high-density lipoprotein cholesterol ratio have been associated with higher levels of salivary estradiol throughout an entire menstrual cycle among premenopausal women, while no such association was observed for progesterone (Furberg et al. 2005). Insulin resistance associated with overweight lowers serum concentrations of SHBG, resulting in an increase in the bioavailable serum estradiol (Key and Pike 1988, Calle and Kaaks 2004). Insulin levels, height and birth weight have been associated with levels of salivary estradiol throughout a menstrual cycle among premenopausal women (Finstad et al. 2009a, Finstad et al. 2009b). However, further studies are needed to fully understand the biological mechanisms involved in linking energy balance and metabolic profile with ovarian responsiveness and hormone synthesis and metabolism.

In general, little or no increased breast cancer risk has been observed according to oral contraceptive use (Ursin et al. 1998, Marchbanks et al. 2002, Neslon et al. 2012) or related to oral contraceptive formulation (Marchbanks et al. 2012). However, a small increase in breast cancer risk has been observed among current users of oral contraceptives (Collaborative group on hormonal factors in breast cancer 1996, Kumle et al. 2002, Lund et al. 2007), among women in the following 1-9 years after stopping use of oral contraceptives (Collaborative group on hormonal factors in breast cancer 1996), among ever users in Iranian young women (Fabre et al. 2007, Ghiasvand et al. 2011), and among women who used oral contraceptives before their first full-term pregnancy (Pike et al. 1981). Studies

focusing on the association between oral contraceptive use and its effect on premenopausal ovarian hormone levels are sparse.

Alcohol consumption equivalent to 3-6 drinks per week has been associated with an approximate 15 % increased risk of breast cancer among both pre- and post-menopausal women (Baan et al. 2007, Chen et al. 2011). Alcohol consumption has also been related to increased levels of ovarian hormones (Reichman et al. 1993), although the results have been contradictory (Dorgan et al. 1994, Tsuji et al. 2012).

1.5.4 Mammographic density

Mammography with low-energy-X-rays is used in screening programs and in clinical practice to examine the female breasts for the presence of precancerous lesions and breast cancer. Mammographic density is also used as an intermediate endpoint in epidemiological breast cancer research and in statistical models for risk prediction of breast cancer. Mammographic density reflects the relative amount of connective and epithelial tissue and fat in the breast (Figure 9) and is the strongest independent risk factors for breast cancer apart from age and sex (Boyd et al. 2005, Palomares et al. 2006, McCormack et al. 2006).

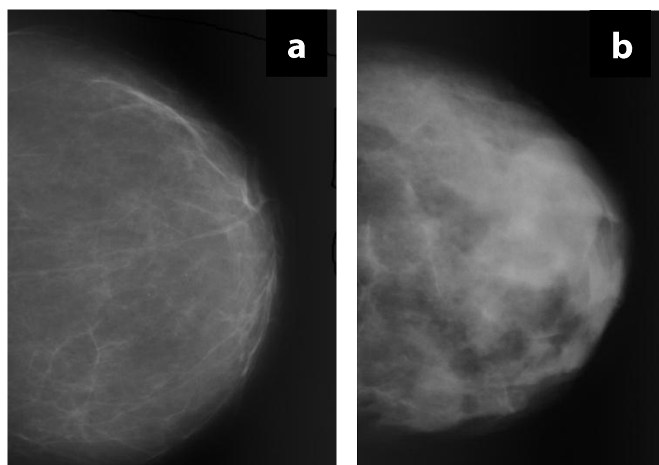


Figure 9. Illustration of mammographic density from the EBBA-I Study.
a) Low mammographic density: the breast is comprised largely of fat which appear black (non-dense).
b) High mammographic density: the whole breast is comprised of dense tissue, representing epithelial and connective tissue which appear white (radiodense).

It has been estimated that 20 % of premenopausal and 10 % of postmenopausal women have a mammographic density above 50 % (McCormack et al. 2006), and mean density in women aged 40-59 years is 18-38 % (Boyd et al. 2002a). The amount of mammographically dense breast tissue has been shown to decline with advancing age (Wolfe 1976, Byrne et al.

1995, Boyd et al. 1998), full-term pregnancy (Gram et al. 1995, Martin and Boyd 2008, Loehberg et al. 2010), and a greater number of births (Boyd et al. 1998, Grove et al. 1985, Martin and Boyd 2008). Furthermore, mammographic density decreases with cessation of menstruation (Grove et al. 1979, Vachon et al. 2000, Boyd et al. 2002a). Body weight has consistently been found to be inversely associated with mammographic density (Brisson et al. 1984, Grove et al. 1985). However, these potential breast cancer risk factors account for only 20-30 % of the variance in mammographic density. Thus, most of the variance in mammographic density is currently thought to be explained by unidentified genetic variance (Boyd et al. 2009, Vachon et al. 2012).

Importantly, benign or precancerous lesions associated with high mammographic density may be fibroadenomas, which are characterized by abnormal growth of glandular and fibrous tissues. Microcalcifications are calcium deposits in the breast tissue that depending on size, distribution, form and density on a mammogram are defined as benign, suspicious or malignant lesions.

Mammographic density can be assessed by qualitative or quantitative methods. In 1976, John Wolfe devised a predominantly qualitative system for characterizing mammographic density based on parenchymal patterns: N1, predominantly fat, no ducts visible; P1, mainly fat, prominent ducts in up to 25 % of the volume of the breast; P2, prominent ducts occupying 25-75 % of the breast volume; and DY, extensive density (Wolfe 1976, Furberg et al. 2005). A more recent qualitative method that classifies parenchyma according to five categories based on anatomic-mammographic correlations was developed by László Tabár (Gram et al. 1997).

Using quantitative measures, the density of a breast mammogram is given as either the percentage of the total breast area, or as the absolute amount of dense area of the breast in cm^2. Norman Boyd introduced a six category classification system where both the density of the total breast area and the amount of fat tissue are considered (Boyd et al. 1995, Boyd et al. 2007). Computer-assisted methods for estimating mammographic density have emerged

to reduce labor input and subjectivity, and to create measures on a continuous scale (Ursin et al. 2003, Boyd et al. 2011, Woolcott et al. 2012, Crandall et al. 2012). However, the methods described above only provide a 2D projection of a 3D breast. Fully automated classification methods and methods taking the breast volume into account are needed and are currently under development (Assi et al. 2011).

Quantitative measures are superior to qualitative measures in breast cancer research (MacCormack et al. 2006). However, it is not clear whether relative (percent) or absolute mammographic density is the best predictor of breast cancer risk. The potential mechanisms for increased breast cancer risk associated with mammographic density are not fully understood. However, since mammographic density represents expression of epithelium among other factors, it is a reflection of the amount of susceptible breast tissue and, in part, the amount of proliferative activity in the mammary glands. Mammary epithelium that is less differentiated has been shown to have a higher proliferation rate, and may be more susceptible to carcinogens and malignant transformation (Russo et al. 1982, DeVita et al. 2011).

Mammographic density has been extensively associated with increased breast cancer risk in both premenopausal and postmenopausal women (Warner et al. 1992, MacCormack et al. 2006). Women with a higher level of mammographic density have a four to six times greater risk of incident breast cancer compared to women with less dense breasts (Brisson et al. 1984, Byrne et al. 1995, Boyd et al. 2007), independent of ethnicity (Ursin et al. 2003). Furthermore, it has been estimated that about one third of all breast cancer cases may be explained by high mammographic density (Byrne et al. 1995, Boyd et al.1995). However, because most premenopausal women included in previous studies of mammographic density were in their late 30s and 40s, it is still not known to what extent mammographic density in younger women is predictive of breast cancer risk later in life (MacCormack et al. 2006).

Studies of endogenous estrogen and progesterone (Greendale et al. 2005, Martin and Boyd 2008) and estrogen plus progestin use among postmenopausal women have shown that hormonal factors influence mammographic density (McTiernan et al. 2005, Greendale et al. 2003). A predictive effect of pubertal height on mammographic density among premenopausal women has also been reported (Lope et al. 2011).

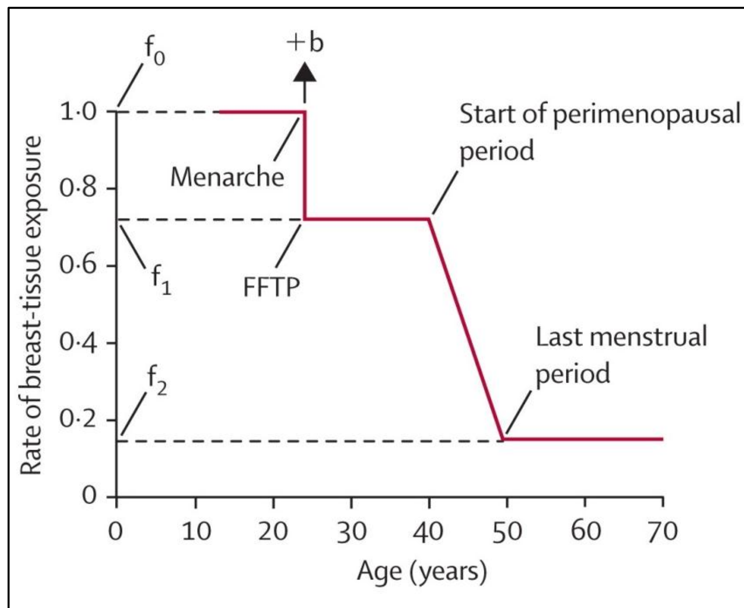


Figure 10. Pike's model of rate of breast tissue aging: FFTP, first full-term pregnancy; b, variable used to calculate age at menarche; f_0, f_1 and f_2 are variables of model. (Reprinted from Pike MC et al. Nature 303:767-770, 1983, with permission from Nature Publishing Group).

This may relate well with Pike's model which suggests that exposure of the breast tissue to hormone-related risk factors throughout life, rather than the chronological age per se, may explain the increased breast cancer risk observed by age despite a decrease in mammographic density (Pike et al. 1983, Boyd et al. 2005). However, knowledge about the association between endogenous ovarian hormones and mammographic density among premenopausal women is limited (Walker et al. 2009, Noh et al. 2006), and inconsistent (Noh et al. 2006, Boyd et al. 2002b).

2 Aims of the thesis

The overall aim of this thesis was to study associations between genetic susceptibility reflected by selected estrogen-related polymorphisms, reproductive factors, lifestyle factors and premenopausal levels of 17β-estradiol and progesterone, and to study associations between these ovarian hormones and mammographic density.

More specifically, the aims were:

- To study whether eight SNPs in *CYP17*, in combination with higher levels of metabolic risk factors, are associated with daily 17β-estradiol and progesterone concentrations in premenopausal women.

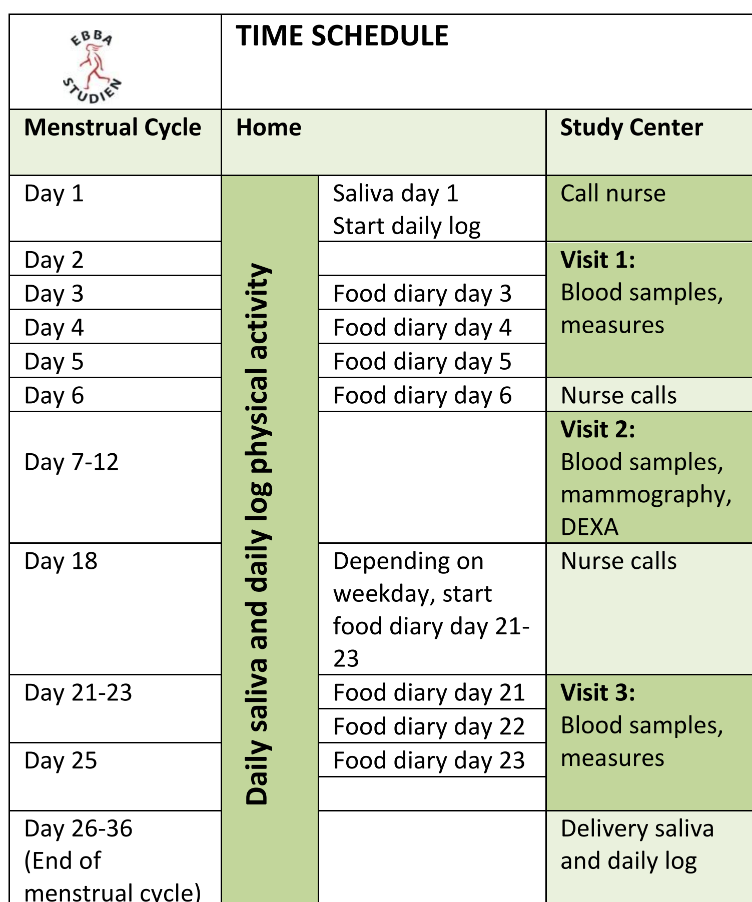
- To study whether parity and the 'menarche-to-first birth' time interval are associated with daily 17β-estradiol and progesterone concentrations in premenopausal women.

- To study whether 17β-estradiol and progesterone concentrations are associated with mammographic density in premenopausal women.

3 Subjects and methods

3.1 Study population and design

The Energy Balance and Breast Cancer Aspects (EBBA)-I Study was designed to explore the association between genetic susceptibility, lifestyle, reproduction, ovarian function and biomarkers of breast cancer risk, including mammographic density among healthy premenopausal women. The EBBA-I Study was conducted between 2000-2002 at the University of Tromsø and the University Hospital of North Norway (UNN), Tromsø, Norway. Women in the municipalities of Tromsø (urban population) and Balsfjord (rural population), North-Norway, were recruited by local announcements in media and public meeting places. Participants were followed throughout one entire menstrual cycle (Figure 11).



EBBA STUDIEN	TIME SCHEDULE		
Menstrual Cycle	**Home**		**Study Center**
Day 1	Daily saliva and daily log physical activity	Saliva day 1 Start daily log	Call nurse
Day 2			**Visit 1:** Blood samples, measures
Day 3		Food diary day 3	
Day 4		Food diary day 4	
Day 5		Food diary day 5	
Day 6		Food diary day 6	Nurse calls
Day 7-12			**Visit 2:** Blood samples, mammography, DEXA
Day 18		Depending on weekday, start food diary day 21-23	Nurse calls
Day 21-23		Food diary day 21 Food diary day 22	**Visit 3:** Blood samples, measures
Day 25		Food diary day 23	
Day 26-36 (End of menstrual cycle)			Delivery saliva and daily log

Figure 11. Time schedule for the EBBA-I Study.

A total of 219 volunteers met the inclusion criteria: age 25-35 years, self-reported regular menstruation, normal cycle length within the previous 3 months, no pregnancy, lactation or use of steroid contraceptives over the previous 6 months, no history of gynecological disorders and no chronic disorders, e.g. diabetes and hypo- or hyperthyroidism. Among the 219 women, 12 women did not complete the study due to: pregnancy, serious illness, accidents, death, disease among family members or relocation. All participants signed a written informed consent at the time of inclusion in the study. The study was approved by the Norwegian Data Inspectorate and recommended by the Regional Committee for Medical and Health Research Ethics, North-Norway. Information about the study population for the three papers in this thesis is summarized in Figure 12.

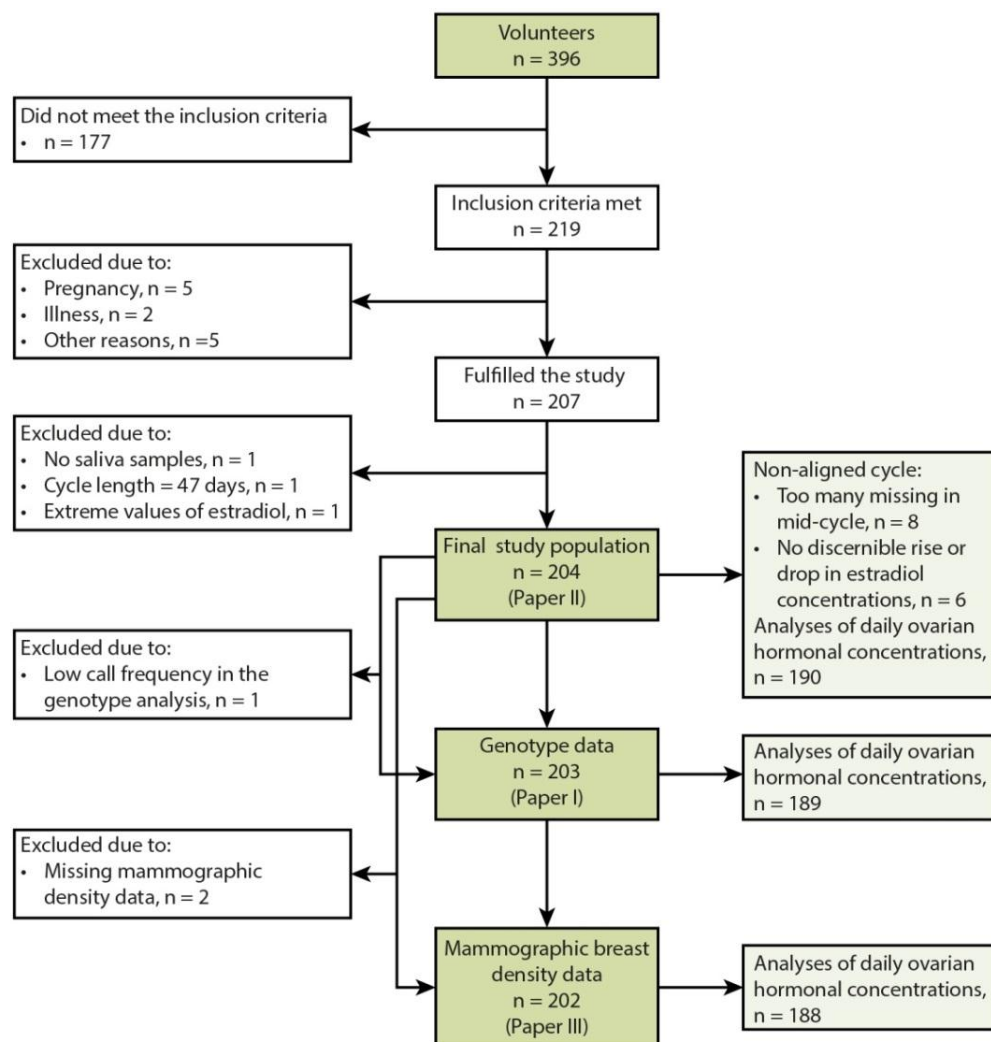


Figure 12. Design of the studies in this thesis.

3.2 Questionnaires: Reproductive and lifestyle factors

Information about demographic variables, ethnicity, reproductive history and past and current lifestyle was collected by a self- and interviewer-administered general questionnaire (appendix). All questionnaires were checked for inconsistencies through face-to face consultation between participants and one trained nurse. In addition, two Medical Doctors [Thune (PI) and Furberg] met all of the participants in clinical settings, and verified all completed questionnaires. To improve recall, a lifetime calendar with examples of milestones was provided for the participants (appendix).

Age at menarche was assessed by the question "How old were you when you had your first menstrual period?" (given in years and months). Parity was assessed by the question "Have you had children?" (if yes, given by consecutive number, year of birth and number of months of breast-feeding for each child). The dichotomous smoking variable used in the three papers in this thesis was assessed by the question "Do you smoke every day now?" (tick yes or no). A continuous variable indicating the number of total alcohol units consumed per week was computed from the reported average number of alcohol units per week [from four types of beverages (Beer, wine, fortified wine, spirits) consumed during the past 12 months] (Nilsen et al. 1992).

A separate questionnaire describing previous hormonal contraceptive use included summary measures (i.e. ever having used, use before first pregnancy) and detailed questions for each period of use (i.e. age when use started and stopped, duration of use and the name of the brand used), which were validated in the Norwegian Women and Cancer Study (Kumle et al. 2002) (appendix). A folder with photos of all the available brands of hormonal contraceptives was enclosed with the questionnaire to help women to recall use of these drugs (Kumle et al. 2002, Lund et al. 2007). Total duration of previous oral contraceptive use, excluding minipills, was used in the statistical analysis in Papers II and III.

Dietary data was collected for seven days during one menstrual cycle (Days 3-6 and Days 21-23) (Figure 11), and a photographic booklet of portion sizes (appendix) was used to help women record the type and portion of every food item consumed 24 hours per day in a pre-coded food diary (appendix) developed for the EBBA-I Study (Kristensen et al. 2004). Also, the participants kept a record of daily saliva sampling, as well as the type and duration of daily physical activity in a logbook (diary) (appendix) designed with contributions from collaborators Bernstein L, Friedenreich C, McTiernan A and Ainsworth B. These data were reviewed for inconsistencies in a face-to face consultation with the trained study nurse.

3.3 Clinical variables: Anthropometry, blood pressure, lipids, glucose, insulin

All participants met at the Clinical Research Center, UNN, for clinical examinations at three scheduled visits during their menstrual cycle (Figure 11): first visit (days 1-5 of the menstrual cycle), second visit (days 7-12), and third visit (days 21-25). All procedures at almost every visit and all quality control of measurements were performed by the same study nurse. At every visit, blood pressure was measured three times with an portable monitoring system (Propaq 102 EL, Protocol systems Inc., Beaverton, OR, USA) (Supplement, Paper I).

Anthropometric measurements were taken at every visit with participants wearing light clothing. Body height was measured to the nearest 0.5 cm, and body weight to the nearest 0.1 kg on an electronic scale. Waist circumference was measured in a horizontal line 2.5 cm above the umbilicus, and hip circumference was measured at the largest circumference of the hip, both to the nearest 0.5 cm (WHO 2011). At the second visit (Days 7-12), the participant underwent a whole-body scan using dual energy X-ray absorptiometry (DEXA) (DPX-L 2288, Lunar Radiation Corporation, Madison, WI, USA) for estimation of the total percentage of fat tissue.

Morning blood samples were taken at each of the three scheduled visits, after a fasting period that started at midnight. Glucose and lipid concentrations were measured in fresh serum at the Department of Clinical Chemistry, UNN, Tromsø, Norway. Serum glucose

was measured enzymatically by the hexokinase method and fasting triglycerides were assayed by enzymatic hydrolysis with lipase. Total cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. High-density lipoprotein cholesterol (HDL-C) was quantified by direct assay using PEG-modified enzymes and dextran sulfate. Glucose and lipids were measured in kits from Roche Diagnostics GmbH, Mannheim, Germany. Insulin was measured in 2003 at the Hormone Laboratory, Aker University Hospital, Oslo, Norway, in serum stored at -70°C until analysis by radioimmunoassay (RIA) (Linco Research Inc., St. Charles, MO, USA). Homeostatic model assessment (HOMA) score [fasting glucose (mmol/l) x fasting insulin (μIU/ml) / 22.5] was used as an indicator of insulin resistance (Keskin et al. 2005). There was no marked drift in any serum variables during the study period at any of the laboratories. The coefficients of variation derived from the laboratories were as follows: 2 % for glucose, 2 % for triglycerides, 2.5 % for cholesterol, 3 % for HDL-C, and 8-12 % for insulin.

3.4 17β-estradiol and progesterone

Participants self-collected daily saliva samples at home, preferentially in the morning, for one entire menstrual cycle, starting on the first day of bleeding (Ellison and Lipson 1999, Furberg et al. 2005). Sampling took place according to previously established protocols developed at the Reproductive Ecology Laboratory at Harvard University, Cambridge, MA, USA, and prior to intake of food, drinks and brushing of teeth. Chewing gum of a special brand was validated and used for a few seconds in order to stimulate saliva production (Lipson and Ellison 1989). Salivary 17β-estradiol concentration was assayed for a total of 20 days (reverse cycle days −5 to −24; with the last day of the menstrual cycle designated −1) and progesterone for a total of 14 days (reverse cycle days −1 to −14) of the menstrual cycle using I-125-based RIA kits (Diagnostic Systems Laboratories, Webster, TX, USA). The salivary assays were done at Harvard University (Furberg et al. 2005). All samples were run in duplicate, and all samples from a single participant were run together in the same assay, with women randomly assigned to assay batches. The sensitivity of the 17β-estradiol assay was 4 pmol/l. Average intra-assay variability was 9 %, and inter-assay variability ranged

from 23 % for low pools to 13 % for high pools. For progesterone, the sensitivity of the assay was 13 pmol/l. Average intra-assay variability was 10 %, inter-assay variability ranged from 19 % for low pools to 12 % for high pools.

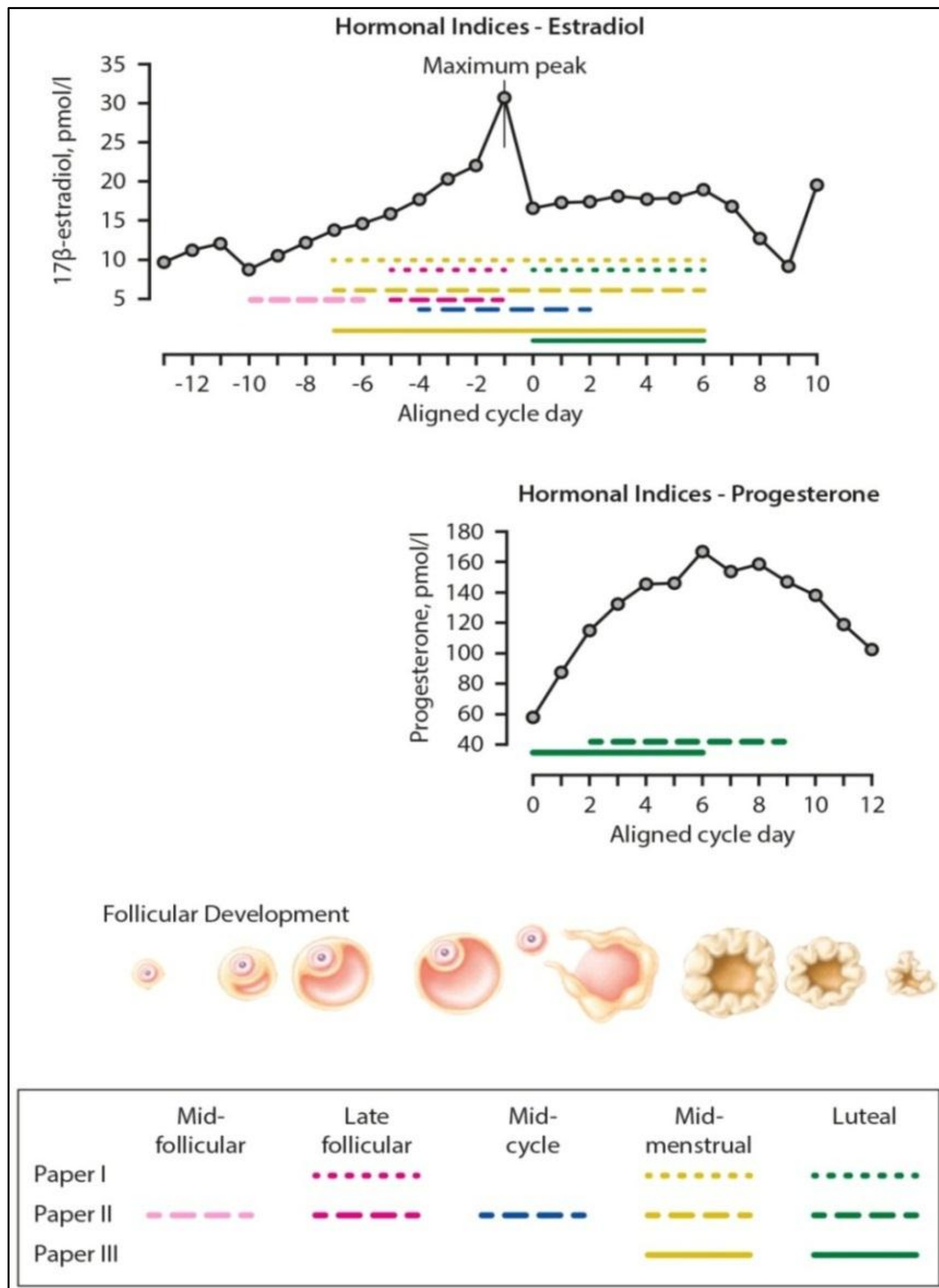


Figure 13. Illustration of hormonal indices for salivary 17β-estradiol and progesterone (Papers I-III).

Prior to statistical analysis of daily ovarian hormone levels, the cycles of the participants were aligned at mid-cycle following published methods (Lipson and Ellion 1996).

Alignment was based on the identification of the mid-cycle drop in salivary 17β-estradiol concentration (aligned cycle day 0), which provides a reasonable estimate of the day of ovulation. Satisfactory identification of the mid-cycle drop in salivary 17β-estradiol concentration could not be made for 14 women, of whom eight had too many missing measurements during mid-cycle, and six had no discernible rise or drop in the concentration of salivary 17β-estradiol during the critical time window. For the remaining 190 women with aligned cycles, follicular and luteal hormonal indices were calculated (Figure 13) (Papers I-III).

Fasting estradiol, progesterone and SHBG concentrations in fresh serum taken at each of the three scheduled visits were measured at the Department of Clinical Chemistry, UNN. Estradiol and progesterone were measured by direct immunometric assay (Immuno-1, Bayer Diagnostics, Norway), with a sensitivity of 0.01 nmol/l and 0.13 nmol/l for estradiol and progesterone, respectively. The coefficient of variation was 3.9 % for estradiol mesurements and 5.7 % for progesterone measurments. SHBG was measured by an immunometric method (Diagnostic Products Corporation (DPC)-Bierman GmbH, Bad Nauheim, Germany) with a coefficient of variation of 5-10 %.

3.5 Genetic biomarker *CYP17* – selection and genotyping

The selection of candidate genes and SNPs was hypothesis driven and made after extensive evaluations of genes related to sex steroid synthesis and metabolism. Altogether, 352 SNPs in 33 genes were successfully genotyped at Fred Hutchinson Cancer Research Center, Seattle, WA, USA. As the EBBA-I samples were genotyped on a platform used in a parallell cancer study at Fred Hutchinson Cancer Research Center, the final list of 352 SNPs extended the original EBBA-I selection. This thesis includes data from a single gene, *CYP17*. DNA from 207 participants was extracted from whole blood stored at -70°C using MagAttract DNA Blood Mini M48 kit (Qiagen, Oslo, Norway) by the Department of Medical Genetics, UNN. To represent the variability of *CYP17* in Caucasians, eight SNPs (rs1004467, rs743575, rs4919687, rs3781286, rs3824755, rs10786712, rs743572, rs2486758) were selected using the Genome Variation Server (LD select) (Carlson et al. 2004). The

SNPs were selected at an r^2 threshold of 0.8 and a minor allele frequency of > 5 %. TagSNP coverage extends 2 kb upstream and 1kb downstream of the gene. Genotyping was performed using the Illumina Golden Gate platform (2008). Twenty-two blinded replicates and a genotype control for the Caucasian population (NA07034) were included in the assay. One sample failed with a call frequency < 85 % and none of the selected SNPs of *CYP17* were monomorphic or significantly out of the Hardy-Weinberg Equilibrium.

3.6 Mammograms and mammographic density

The participants had bilateral mammograms taken during the late follicular phase (days 7-12) at the Center for Breast Imaging, UNN. The mammograms were craniocaudal (from above a horizontally compressed breast) and mediolateral-oblique (from the side and at an angle of a diagonally compressed breast) using Siemens Mammomat 3000 (Furberg et al. 2005).

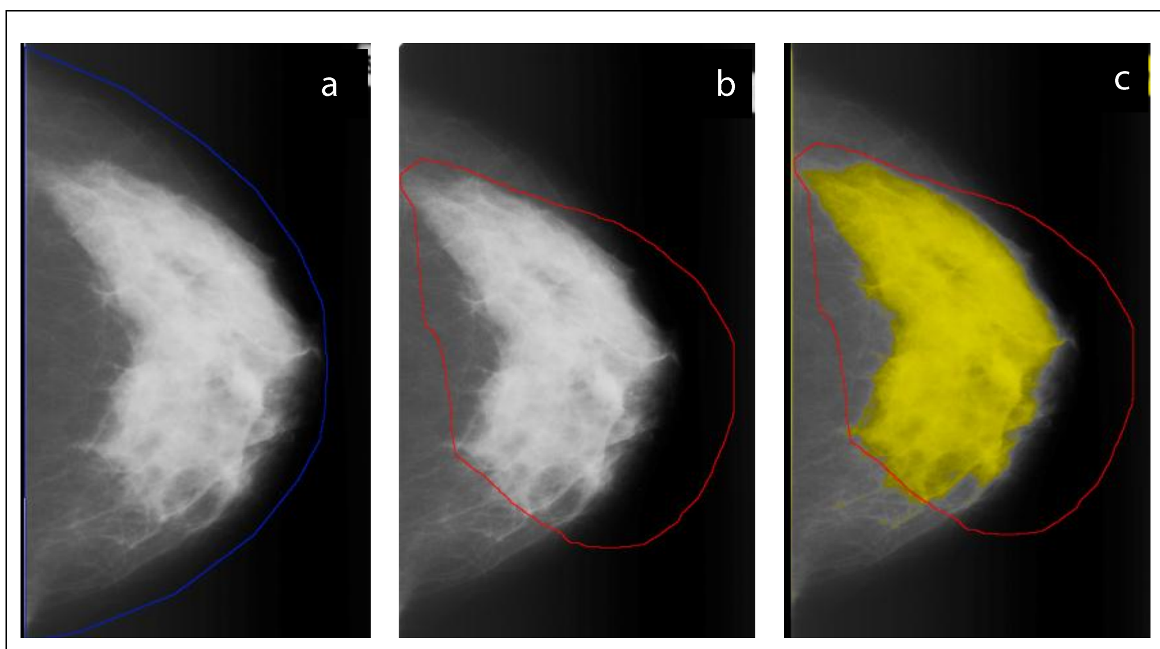


Figure 14. Illustration of assessment of mammographic density by MADENA: a) the total breast area (blue line) is calculated by the software (here: 147.20 cm^2), b) a region of interest is marked (red line) by the reader around areas considered to contain mammographic densities, c) a threshold for mammographically dense areas is set (yellow). The size of the yellow area is estimated by the computer (here 58.68 cm^2). Percent density is calculated (100 % x 58.68cm^2 / 147.20cm^2 = 39.9 %) (Reprinted from Ursin G and Qureshi SA. Norsk Epidemiol 19:59-68, 2009).

The left craniocaudal mammograms were digitized in 2011 using a high-resolution Kodak Lumisys 85 scanner with automatic feeder (Kodak, Rochester, NY, USA) and imported into a computerized mammographic density assessment program (MADENA) developed at the University of Southern California, School of Medicine Los Angeles, CA, USA (Ursin et al. 2003). The total breast area was defined on the mammographic image by a research assistant trained by Ursin using a special outlining tool (Figure 14a). Furthermore, the region of interest (ROI), excluding the pectoralis muscle, prominent veins and fibrous strands, was defined (Figure 14b). The experienced mammogram reader (Ursin) used a tinting tool to apply yellow tint to pixels considered to represent areas of mammographic density (Figure 14c).

The MADENA software estimated the total numbers of pixels and the number of tinted pixels within ROI. Absolute mammographic density represents the count of the tinted pixels within ROI, and percent mammographic density is the ratio of absolute density to the total breast area (area of ROI) multiplied by 100. The mammograms were read in four batches with equal numbers of mammograms in each batch. A duplicate reading of 26 randomly selected mammograms from two of the batches showed a Pearson's correlation coefficient of 0.97. The reader was blinded to any characteristics of the study population.

3.7 Statistical methods

Statistical analyses were performed using STATA version SE 11.0 (Stata Corporation, Cellege Station, TX, USA). In addition, SNPStats free internet software based on algorithms from haplo.stats (software R, server hosted by the Institute for Statistics and Mathematics of the Wirtschaftsuniversität, Vienna, Austria), was used for the haplotype analysis (Paper I). Two sided p-values < 0.05 were considered statistically significant. Hormone data were log transformed prior to statistical analyses in order to fulfill the model assumptions of normal distribution.

We constructed a clustered metabolic score by summarizing z-scores [(individual raw values – sample mean) / sample standard deviation] of waist circumference, fasting

triglycerides, total cholesterol / HDL-C ratio, homeostatic model assessement (HOMA) score (Wallace and Matthews 2002), and mean arterial pressure [(2 diastolic blood pressure + 1 systolic blood pressure) / 3] (Paper I). HOMA score and fasting triglycerides were log transformed prior to the calculation of z-scores. This score was constructed based on metabolic scores associated with physical activity, physical fitness (Thune et al. 1998, Rizzo et al. 2007, Emaus et al. 2008b), ovarian hormones (Furberg et al. 2005, Emaus et al. 2008a), breast cancer risk (Thune et al. 1997, Furberg et al. 2004, Bjørge et al. 2010) and survival (Emaus et al. 2010a), and the definition of metabolic syndrome (International Diabetes Federation) (Alberti et al. 2006). This metabolic score was considered as a continuous variable with a mean of 0, so that lower values corresponded to a more favorable profile. Exploratory factor analysis was used to describe the correlations among the variables in the clustered metabolic score, and to determine the number of factors describing the covariance structure (Tinsley and Tinsley 1987). Kaiser's criterion (eigenvalue > 1) and Cattell's scree plot were used for factor extraction. The exploratory factor analysis indicated a satisfactory loading (> 0.400) for all variables except fasting triglycerides (< 0.400). Fasting triglycerides were nevertheless kept in the clustered metabolic score because of biological plausibility.

See also Papers I-III for further description of statistical methods.

4 Main results

Paper I

Genetic polymorphism *CYP17* rs2486758 and metabolic risk factors predict daily salivary 17β-estradiol concentration in healthy premenopausal Norwegian women. The EBBA-I Study

Among premenopausal women participating in the EBBA-I Study, we observed that having the *CYP17* rs2486758 minor allele genotype was associated with an 18.5 % higher overall concentration and an 18.0 % higher luteal phase concentration of salivary 17β-estradiol compared with having a homozygote genotype of the major allele. Moreover, women with the minor allele and levels of metabolic risk factors in the upper tertile had higher daily salivary 17β-estradiol concentrations: 24 % higher for HOMA score, 32 % higher for fasting triglycerides, 44 % higher for total cholesterol / HDL-C ratio, and 53 % higher for clustered metabolic score, compared with all the other women combined. When comparing women in the upper tertile of the metabolic risk factors we found that having the minor allele was associated with 29 % higher daily salivary 17β-estradiol concentration for fasting triglycerides, 35 % for total cholesterol / HDL-C ratio, and 38 % for clustered metabolic score, compared with women having the homozygote genotype of the major allele. Our results suggest that genetically susceptible women with the *CYP17* rs2486758 minor allele may benefit from interventions aimed at modifying metabolic risk factors for the purpose of lowering estradiol levels and prevention of breast cancer.

Paper II

Ovarian hormones and reproductive risk factors for breast cancer in premenopausal women: the Norwegian EBBA-I Study

In the second paper from the EBBA-I Study, we observed no association between parity and overall salivary concentrations of 17β-estradiol or progesterone. Compared with nulliparous women with smaller waist circumference (< 77.75 cm) or shorter duration of previous oral contraceptive use (< 3 years), nulliparous women with larger waist circumference (≥ 77.75 cm) or longer durations of previous oral contraceptive use (≥ 3 years) were both associated with higher salivary levels of 17β-estradiol across the menstrual

cycle. The 'menarche-to-first birth' interval was inversely associated with overall mean salivary concentrations of 17β-estradiol (P_{trend} = 0.029). This inverse relationship was observed across different groups according to weight, age, and age at menarche. Short (< 10 years) versus long (> 13.5 years) 'menarche-to-first birth' interval was associated with 47 % higher maximum peak and 30 % higher mid-cycle levels of daily salivary 17β-estradiol. We observed a 2.6 % decrease in overall average salivary 17β-estradiol with each 1-year increase in the interval. No associations between the 'menarche-to-first birth' interval and salivary progesterone concentrations were observed. Our results suggest that lifestyle factors including previous oral contraceptive use and excess abdominal adiposity are associated with higher levels of estradiol, and that nulliparous women may be more susceptible to the effects of these lifestyle factors on estradiol across the menstrual cycle.

Paper III

Associations of daily 17β-estradiol and progesterone with mammographic density in premenopausal women. The Norwegian EBBA-I Study

Among 202 women in the EBBA-I Study, we observed a mean percent mammographic density of 29.8 %. Compared with women with mammographic density < 28.5 %, women with mammographic density ≥ 28.5 % had 25 % higher daily salivary concentration of 17β-estradiol (P = 0.007), and 31 % higher daily salivary progesterone concentration (P = 0.010) across the menstrual cycle. Compared with women in the first quartile of overall average salivary concentrations of 17β-estradiol and progesterone, the women in higher 17β-estradiol and progesterone quartiles had greater odds of higher percent mammographic density (≥ 28.5 %) (17β-estradiol Q4 vs. Q1: OR 2.69, 95 % Confidence interval (CI) 0.97-7.51, P_{trend} = 0.031; and progesterone Q4 vs. Q1: OR 3.70, 95 % CI 1.35-10.11, P_{trend} = 0.011). These associations were even stronger among nulliparous women. We also observed strong associations between serum concentrations of ovarian hormones and percent mammographic density. Our results suggest that daily 17β-estradiol and progesterone are strongly associated with percent mammographic density in premenopausal women.

5 Discussion

The EBBA-I Study is the largest study of its kind and provides unique data on day-to-day ovarian hormone profiles in a sample of 204 young women. The high-quality estimates of ovarian hormone levels and the high retention and completeness of data are the greatest strengths of this study. Moreover, genotyping of carefully selected candidate genes and SNPs was performed to test whether polymorphisms in genes coding for key enzymes in the steroidogenic pathway are associated with biomarker concentrations in saliva and serum. In addition, percent mammographic density was assessed by the more accurate computer-assisted method MADENA. Before we draw conclusions from our findings we must consider some basic questions regarding the selection of the study population and discuss to what extent the observed associations may result from bias, confounding or chance, and to what extent they may be related to biological mechanisms in breast cancer development. The relatively small number of women in subgroups may limit the statistical power. As a result, associations in subgroups could not be adequately elucidated.

5.1 Methodological considerations

Systematic errors or bias may arise from a consistent fault in the design, conduct or analysis of a study that results in mistaken estimates of the observed value that is hard to correct for. It is important to achieve results that are minimally affected by systematic bias since the degree of systematic bias defines the validity of a study. Systematic bias may be discussed in two perspectives; internal validity (i.e. selection bias, information bias and confounding) and external validity (i.e. selection bias, generalizability).

5.1.1 Internal validity

Selection bias

Selection bias may occur from procedures used to select subjects and factors that influence study participation so that the association between exposure and outcome differs between the study subjects and those who do not participate in the study (Rothman 2002). The subjects described in this thesis volunteered for participation in the EBBA-I Study. These

women might have been especially health conscious which could have affected their lifestyle (e.g. energy intake, use of hormonal contraceptives) and also their willingness to participate in the study. This self-selection may also have led to an overrepresentation of women with a familial history of breast cancer or women with a particular interest in undergoing clinical examinations or mammography, resulting in participants that differ from non-participating women. However, data from the questionnaires regarding family history of breast cancer (not shown in this thesis) indicated that 10 % of the participants had a familial history of breast cancer, which is about the same as in the general Norwegian population (Cancer Registry of Norway). Among our study participants, mean age at menarche was 13.1 years, which is comparable to the observed mean age at menarche of 13.0 years among 74 973 women surveyed at the same age in another Norwegian study (Bjelland et al. 2011). Furthermore, we observed comparable mean values for measures such as BMI and serum cholesterol as in women (30-39 years) from the general population of Tromsø (The Fifth Tromsø Study), and in Norway (Norwegian Institute of Public Health). Therefore, it is not likely that the participants in our study differ significantly from the general Norwegian female population of the same age-group with respect to reproductive and metabolic risk factors. However, the associations between exposures and outcomes among non-participating women are not well known for all variables. Thus, it will be hard to exclude the possibility of some differences between our participants and the non-participating women.

Information bias and misclassification

Information bias refers to bias that is related to instruments and techniques used to collect information about exposure and outcome variables. Differential misclassification may occur if the misclassification of exposure differs by the outcome status (Rothman 2002). This can affect the associations in both directions, leading to spurious associations. The participants in the EBBA-I Study did not know their level of ovarian hormones or their mammographic density (outcome variables). Thus, it is unlikely that their questionnaire responses could have been influenced by their outcome status. Non-differential

misclassification may occur when all groups or categories of a variable (exposure, outcome, or covariate) have the same error rate or probability of being misclassified for all study subjects. Usually non-differential misclassification dilutes the effect of the exposure.

In our analyses, we considered salivary ovarian hormones through the entire course of a menstrual cycle as both an exposure (Paper III) and as a major outcome (Papers I-III). Salivary steroid levels reflect the free (unbound to SHBG, CBG and albumin) and biologically active fraction of hormones in blood (Ellison and Lipson 1999, Chiappin et al. 2007) and the daily sampling regime provides detailed characterization of hormonal menstrual cycle variability (Campbell and Ellison 1992, Ellison 1993a). The use of salivary steroid analysis has been pioneered in the laboratory by Peter T. Ellison (Ellison and Lager 1986, Lipson and Ellison 1989, O'Rourke and Ellison 1993, Ellison and Lipson 1999, Ellison et al. 1993b, Jasienska and Thune 2001, Furberg et al. 2005) and is an innovative technique in breast cancer research. Thus, concentrations of sex steroids in saliva are regarded as a valid estimate of ovarian function (Jasienska and Jasienski 2008).

Saliva samples can easily be stored without refrigeration since steroid levels remain stable in samples stored in room temperature for several months (Lipson and Ellison 1989). In our study, a minor number of samples (n = 29, 14 %) of salivary 17β-estradiol measurements were conducted with an assay replaced by a new method. Although this change of method might have introduced a non-differential misclassification to the salivary 17β-estradiol measurements, parallel runs between the assays showed a correlation comparable to what the laboratory would expect for a sample ran twice with the same method.

Mammographic density was used both as an exposure and an outcome variable in Paper III. By utilizing mammographic density on the continuous scale, it is easier to detect small effects that may be harder to elucidate using qualitative measurements of mammographic density. Determining mammographic density using a more precise computer-assisted method (MADENA) is still dependent on a subjective assessment by the reader. The mammograms in our study were read by one experienced reader (Ursin) blinded to the

characteristics of the participants, thus minimizing the risk of non-differential misclassification. Also, our reader performed a duplicate reading with randomly selected mammograms that showed high correlation (Pearson's correlation coefficient = 0.97). Although a reproducible measure does not necessarily indicate that it is valid, our estimates of association between i.e. age, parity, BMI and mammographic density show patterns of variation in mammographic density (Paper III) also found by others (Samimi et al. 2008, Dorgan et al. 2012). Moreover, the assessment of mammograms in the same narrow time frame during the follicular phase of the menstrual cycle (between days 7-12 after onset of the menstrual cycle) for all women strengthens the validity of our results (Ursin et al. 2001, Morrow et al. 2010, Miglioretti et al. 2011).

In order to minimize non-differential misclassification for the exposure variables assessed by the general questionnaire in the EBBA-I Study, a lifetime calendar and a list of example milestones were presented to the participants to improve their recall. Furthermore, birth records (including birth weight and birth length) from the Norwegian Medical Birth Registry of Norway were obtained in 2006 from 176 of the participating women. Mean birth weight in the national registry was 3366 grams (standard deviation 530 grams, range 1320-4470 grams) compared with a mean self-reported birth weight of 3389 grams (standard deviation 560 grams, range 1380-4946 grams), representing an overall Pearson's correlation between self-reported and recorded birth weight of 0.93 ($P < 0.01$). This is an example of this study population's strong recall, which increases the validity of the data assessed by the questionnaire. Furthermore, recalled age at menarche was almost the same as the average age at menarche among 74 973 same-aged women participating in another study in Norway (Bjelland et al. 2011).

For the assessment of self-reported previous hormonal contraceptive use, a folder with photos of all the available brands of hormonal contraceptive has been developed and validated for use in Norway (Kumle et al. 2002, Lund et al. 2007). This folder was enclosed with the study questionnaire to help women recall use of specific hormonal contraceptives. Association between oral contraceptive use assessed by a similar questionnaire and risk of

breast cancer was observed among 103 027 premenopausal women (Kumle et al. 2002). In addition, women using hormonal contraceptives 6 months prior to the study were excluded from the EBBA-I Study.

Assessment of alcohol intake was performed using validated questions in comparable populations (Nilssen et al. 1992), and was calculated from a numeric variable indicating the number of alcohol units consumed per week. In general, alcohol intake is underreported (Stockwell et al. 2004), in particular among heavy drinkers (Northcote and Livingston 2011). Biomarkers that specifically define alcohol intake have not been fully detected, and the self-reported alcohol intake assessed in the present study is still considered a gold standard (Nilssen et al. 1992); however, some non-differential misclassification is possible.

Assessment of self-reported recreational physical activity was done by using a four level scale originally developed by Saltin and Grimby (1968) and used in the Three Counties Cohort Study as well as in the Tromsø study (Thune et al. 1997, Thune et al.1998, Emaus et al. 2010a). The scale provides valid estimates of high-intensity leisure activity (Thune et al. 1998, Emaus et al. 2010b) and inactivity (Rødjer et al. 2012). Thus, we consider the data from this four-level scale to be adequate for this study.

High reproducibility of the serum measurements of ovarian hormones, SHBG, lipids, glucose, insulin and the genetic analysis was ensured through well-developed protocols with continuous quality controls. These included the use of internal standards and inter-laboratory comparisons; furthermore, several of the serum methods were used in routine hospital analysis. DNA extraction from the blood samples and storage were optimal; however, errors in genotyping assays are not untypical and may lead to biased results (Palmer et al. 2011). Importantly, the genotyping was performed using the Illumina Golden Gate platform by an expert laboratory at Fred Hutchinson Cancer Research Center, Seattle, WA, USA. Twenty-two blinded duplicates and a genotype control for the Caucasian population (NA07034) were included in the assay. The concordance for the blinded duplicate samples and control was 100 %. Only one sample failed with a call rate < 85 %,

and none of the selected SNPs of *CYP17* were monomorphic or significantly out of Hardy-Weinberg Equilibrium. Overall, the laboratory reported results for the genotyping assay as robust or better than average, which strengthens the validity of the genotyping results in this study.

Waist circumference was measured in a horizontal line 2.5 cm above the umbilicus which in young, healthy, mostly non-obese women as in the EBBA-I Study, corresponds to the level of the last rib as specified in the waist measurement procedure of "Anthropometric standardization reference manual" (Lohman et al. 1988). This method may introduce non-differential classification among older and/or obese women. Waist circumference measurements made at the level of umbilicus may underestimate the true waist circumference (WHO 2011), thus we cannot rule out the possibility that waist circumference was underestimated using our method.

Confounding

Confounding is defined as a factor that mixes or blurs the observed effect (Rothman 2002), and may bias the observed estimates if it is associated both with the exposure of interest and the outcome (Rothman 2002). In contrast to selection- and information bias, confounding can be controlled for in the study design by random selection and restriction, or in the statistical analysis by stratification and multivariable analysis. In the EBBA-I Study we limited confounding through specific inclusion criteria including self-reported regular menstruation and normal cycle length, and through exclusion of women with endocrine disorders, recent pregnancies, breast feeding, or use of hormonal contraceptives. This restriction may have increased internal validity, but simultaneously could have weakened the external validity by selecting women that do not fully represent premenopausal women between the ages of 25 and 35 years in the general population. For example, one of the exclusion criteria was use of oral contraceptives over the last 6 months. Excluding these women was necessary when studying ovarian hormones and ovarian function. However, in

general, almost 40 % of women in the same age-group used oral contraceptives during the same time period (Skjeldestad 2007).

In recognition of potential confounding factors, subgroup analyses and multivariable analyses were performed. However, in stratified statistical models real associations can be missed because of inadequate statistical power, and reported associations may be spurious because of the performance of multiple statistical tests. We will argue that it is important to aim at building statistical models that include relevant factors but with as few covariates as possible to make the models more stable, and the estimated standard error as small as possible. However, important confounders as age, BMI and parity must be adjusted for when studying mammographic density (Paper III). Nonetheless, some confounders such as genetic susceptibility could not be elucidated properly due to small sample size; confounding by other unknown factors could also not be ruled out.

5.1.2 External validity

External validity refers to the generalizability of the results of the study. Selection bias could also affect the external validity of a study if the selected participants are different from the corresponding group of people in the general population. Our strict inclusion criteria ensured uniformity regarding health status and factors that could affect ovarian function. The similarities in reproductive factors (Norwegian Institute of Public Health, Bjelland et al. 2011) between participating women and same-aged women in Norway make us confident that new knowledge from this study can be applied beyond this specific group of women. As discussed in the previous section (5.1.1), age at menarche, BMI and components of the metabolic profile were, on average, the same in our study population as in women from the same geographical area as well as in the general population in Norway (The Fifth Tromsø Study, Norwegian Institute of Public Health).

Timing of ovarian hormone measurements and the assessment of mammographic density in relation to menstrual cycle phase is important to ensure external validity (Ursin et al. 2001, Morrow et al. 2010, Miglioretti et al. 2011). Salivary ovarian hormone concentrations

measured across a single menstrual cycle were used as a "hormonal profile" for each participating women. Some may argue that this could threaten external validity since the concentrations of ovarian hormones are known to vary between cycles in premenopausal women. However, a study with repeated hormone measurements during two consecutive menstrual cycles in 12 women, showed higher variance in mean estradiol concentrations between the women than between the cycles from the same woman. This supports the use of a single menstrual cycle as a reasonable estimate of ovarian hormone differences between women (Gann et al. 2001). The curves describing ovarian function in the study population (figures Papers I-III), resemble those observed in the general population (Welt et al. 1999), and correspond with the established physiology of the female reproductive system (Speroff and Fritz 2005). The mean salivary levels of estradiol and progesterone correspond well with reported levels among women of the same age from comparable countries (Jasienska and Thune 2001, Jasienska et al. 2006), and strengthen the validity of our study.

Furthermore, *CYP17* rs2486758 has been genotyped in the international HapMap project and the distribution of genotypes for this SNP in the EBBA-I Study population is similar to other populations with European ancestry (dbsSNP, HAPMAP, Carlson et al. 2004) which supports the external validity of our findings.

Although histopathological findings have been related to mammograms, it is still not fully clear what mammographic density represents biologically. Mammographic density has been associated with some markers of epithelial growth (Guo et al. 2001, Hawes et al. 2006) and with breast stroma (Warren et al. 2003). Thus, mammographic density may reflect precancerous lesions and patterns relevant in breast cancer development, even if the details remain unclear. Currently, women from 50 to 69 years of age are invited to participate in The Norwegian Breast Cancer Screening Program. Thus, few reports of mammographic density from healthy premenopausal women are available in Norway. Yet, in our study, 18 % of premenopausal women had mammographic density greater than 50 %, which is comparable to the estimated 20 % of premenopausal women having mammographic density greater than 50 % in a previous meta-analysis (McCormack et al. 2006). Also, the

assessed mammographic density in our study was negatively associated with age, BMI and parity as reported by others (Samimi et al. 2008, Dorgan et al. 2012).

Previously, in the EBBA-I Study we have reported a crude positive association between average salivary concentration of progesterone, but not with estradiol, and mammographic density assessed qualitatively using modified Wolfe's classification system in a sub-analysis of healthy premenopausal women (Furberg et al. 2005). The computer-assisted mammographic density assessment (MADENA) used in this thesis provides continuous measures that are more precise (McCormack et al. 2006) and allowed us to perform more detailed studies of the association between ovarian hormones and mammographic density. The results included in this thesis emphasize the importance of considering different methods for the assessment of mammographic density when evaluating the relationship between ovarian hormones and premenopausal mammographic density in order to achieve external validity (Paper III). Despite being a simplified, two dimensional measure of a three dimensional breast, the computer-assisted method has been deemed to provide an adequate measure of mammographic density for breast cancer research.

5.2 Discussion of main results

Systematic errors such as selection bias, information bias or confounding do not explain our main findings in the EBBA-I Study. Random errors occur by chance, but can be controlled for by including a sufficient number of observations in the study sample, and in the analyses performed to test the study hypothesis. A sample size of 200 women in the EBBA-I Study gives an 80 % probability of detecting a real difference between two subgroups of at least 2.2 pmol/l and 60 pmol/l in overall average salivary concentrations of 17β-estradiol and progesterone, respectively. Thus, we do not find it likely that our results occurred by chance. The extent to which the observed associations support causality may be judged based on the Bradford-Hills criteria: temporal sequence, strength of association, consistency of association, biological gradient, specificity of association, biological

plausibility, coherence with existing knowledge, experimental evidence, and analogy (Hill 1965).

In the EBBA-I Study, information about both exposure and outcome variables was obtained simultaneously within a narrow time window (one menstrual cycle). However, some exposures such as age at menarche, age at first birth, parity and genotype reflect earlier exposure and may support a temporal relationship with ovarian hormone levels. However, the lack of repeated assessments of the exposure and outcome variables still limits our ability to infer causality. Bearing in mind this limitation, the observed associations will be carefully discussed using Hill's criteria for causal relations when appropriate.

5.2.1 Genetic susceptibility- *CYP17* and ovarian hormones

In our study, the *CYP17* rs2486758 minor allele was associated with 18 % to 53 % higher concentrations of estradiol depending on which metabolic risk factors were studied. A non-significantly higher concentration of progesterone was observed among women with the *CYP17* rs2486758 minor allele compared to women with the *CYP17* rs2486758 major allele.

Genetic susceptibility and gene-lifestyle interactions may contribute to breast cancer development (Becher et al. 2003, Giarelli et al. 2005, AICR 2007, Cerne et al. 2011, Palmer et al. 2011, Huang et al. 2012, Nelson et al. 2012). However, few reports have evaluated the link between genetic and lifestyle factors and endogenous ovarian hormone concentrations among premenopausal women. To our knowledge, there are no prior studies of the *CYP17* rs2486758 in relation to levels of ovarian hormones among women. Recently, selected gene polymorphisms related to estrogen metabolism have been observed to affect breast density and breast cancer risk and survival (Cribb et al. 2011, Johnson et al. 2012, Lee et al. 2012, Ghoussaini et al. 2012, Butt et al. 2012), while an increased breast cancer risk has been observed in *CYP17* rs743572 minor allele carriers (Chakraborty et al. 2007). However, several studies have reported conflicting results and questioned whether *CYP17* genotypes or other genes in the estrogen pathway are related to breast cancer susceptibility (Mitrunen et al. 2000, Canzian et al. 2010, Beckmann et al. 2011). Interestingly, increased levels of

salivary and serum estradiol have been observed among healthy premenopausal women with the hetero- and homozygote genotype of the *CYP17* rs743572 minor allele (Feigelson et al. 1998, Small et al. 2005). This association has also been observed by our research group (Jasienska et al. 2006), but not by others (Garcia-Closas 2002). Inconsistent results have also been obeserved between *CYP17* rs743572 minor allele and serum progesterone (Feigelson 1998, Garcia-Closas et al. 2002).

Importantly, in a larger study, no association between *CYP17* rs2486758 and breast cancer risk was observed (Stetiawan et al. 2007). *CYP17* rs2486758 is localized in the intergenic section near the 5' of *CYP17*, and approximately 40 % of trait-associated SNPs have been found in intergenic regions (Maonolio 2010). Based on current knowledge in this field, we can predict that the *CYP17* rs2486758 minor allele may increase *CYP17* expression; either by affecting gene splicing, transcription factor binding, or the sequence of non-coding RNA (Manolio 2010). However, this estrogen-associated SNP may not be the causative variant itself, but rather point toward a functional genetic variant. The tagSNP rs2486758 was the only SNP in its bin (Supplemental, Paper I), and several databases and the Genome Variation Server were used to obtain the minimal set of SNPs for coverage of variation on *CYP17* (dbsSNP, HAPMAP, Carlson et al. 2004). Extensive sequencing of an associated region may identify additional rare variants (frequency < 5 %) with a possible biologic role. Changes in the feedback sensitivity and adjustment of the estradiol set-point driven by *CYP17* could be a possible explanation for the observed increase in circulating estradiol concentrations in our study; however, this will need to be tested in future studies.

In the present analysis, *CYP17* rs2486758 was not associated with metabolic risk factors and levels of SHBG. Thus, our data support a true gene-environment interaction, in which only premenopausal women with the *CYP17* rs2486758 minor allele are susceptible to the possible estrogen-enhancing effects of a high clustered metabolic score. The observed interaction between *CYP17* and metabolic risk factors in the present study may be particularly relevant as breast cancer development seems to cluster in a subset of the female

population (Balmain et al. 2003), and gene-environment interaction including susceptibility for unfavorable metabolic profiles are plausible mechanisms for breast cancer.

Several tests were performed without correcting for multiple testing when studying the genetic polymorphism *CYP17* rs2486758. Although we understand that this may have increased the chances of obtaining a significant association, we argue that there are several reasons to not to perform a statistical correction for multiple testing. Our study is strictly hypothesis-driven; *CYP17* is a candidate gene with a specific role in the pathway of estrogens and progesterone biosynthesis, and the clinical variables were carefully chosen based on biological plausibility and in accordance with available literature. Unfortunately, by reducing the type I error for null associations (primary goal in multiple testing), the risk of type II error increases.

Salivary ovarian hormone concentrations are a well-defined and validated outcome. We examined estrogen concentrations across the entire menstrual cycle by *CYP17* rs2486758 genotype according to tertiles of the metabolic score (Paper I). The metabolic score was indirectly validated according to physical activity and heart rate in the EBBA-I Study population (Emaus et al. 2008b). Thus, we find that when exploring cross-sectional associations in our study, this is a sound measure of metabolic risk.

5.2.2 Reproductive risk factors and ovarian hormones

We observed no overall association between parity and ovarian hormones, in agreement with former studies (Verkasalo et al. 2001). Among nulliparous women, larger waist circumference and longer use of oral contraceptives prior to the study inclusion were associated with higher levels of estradiol. This suggests that ovarian function may be particularly susceptible to these lifestyle factors before first full-term pregnancy, which may be important for breast cancer risk. Consistent with our finding, a positive linear relationship between body fat and estradiol levels across an entire menstrual cycle was observed in the Polish EBBA study (Ziomkiewicz et al. 2008). Moreover, in a longitudinal study, larger waist circumference in premenopausal women was a predictor of higher

estradiol levels during the menopausal transition (Wildman et al. 2012). Thus, adiposity in young women may initiate prolonged changes in sex hormone concentrations. Accumulation of excessive abdominal fat may be associated with insulin resistance and hyperinsulinemia. Insulin stimulates ovarian steroidogenesis and inhibits the hepatic synthesis of SHBG, leading to increased levels of free estradiol (IARC 2002, AICR 2007, Finstad 2009b). This may explain the positive association between waist circumference and free estradiol in our study. Our findings are contrary to other studies that have reported inverse associations between waist circumference and total estradiol and its main binding protein, SHBG. However, adjustment for serum SHBG measured at the first visit did not change our estimates. Thus, we hypothesize that long-term positive energy balance reflected by larger waist circumference, may increase levels of free estradiol in regularly cycling women, particularly among those that are nulliparous. These hormonal changes possibly induced by abdominal fat may also be of importance for estrogen levels later in life (Wildman et al. 2012).

Our finding of a positive association between previous use of oral contraceptives and biologically active and free estradiol concentrations across a menstrual cycle among nulliparous premenopausal women is poorly documented by others. However, our findings suggest that ovarian function in nulliparous women may be more susceptible to long-term suppression by exogenous hormones and possible boosting of estradiol production after cessation of the pill. Thus, we hypothesize that exposure to oral contraceptives may change the physiological set point for the regulation of endogenous hormone levels, particularly among women that have not experienced a full-term pregnancy.

We observed that parous women with shorter 'menarche-to-first birth' interval had higher parity, lower age at first birth, and higher salivary estradiol levels than women with longer interval. The inverse association between the 'menarche-to-first birth' interval and salivary estradiol concentrations was observed in a dose-response manner. Furthermore, women with the shortest 'menarche-to-first birth' interval had in average 47 % higher maximum peak levels of salivary estradiol concentrations compared with women with the longest

interval. Our results are partly supported by former reports showing that early age at menarche is associated with higher estradiol levels (Bernstein et al. 1991, Emaus et al. 2008a) and higher frequencies of ovulation (Apter et al. 1989). Higher follicular levels of estradiol have been observed in healthy women's menstrual cycles resulting in conception compared with cycles without conception (Lipson and Ellison 1996, Venners et al. 2006). Furthermore, elevated estradiol concentrations may lead to more frequent sexual activity, thereby increasing the likelihood of fertilization and parity (Cutler et al. 1986, Durante and Li 2009). In our study, early age at first birth is the main determinant of a shorter 'menarche-to-first birth' interval rather than late age at menarche, suggesting that either a conscious choice, higher fecundity, or both, may influence the length of the interval.

On the basis of our observations, we hypothesize that the childbearing pattern (i.e. delayed childbirths) in this female population is partly determined by variation in fecundity (beside socio-cultural aspects) which may, in part, be determined by variation in protein coding genes involved in the regulation of the ovarian function as well as gene-environment interactions (Paper I). We did not include socio-economic status (education, professional experience and income) in our analysis as we consider these data as merely proxy estimates correlating with other variables which probably exert the true effect on the outcome. These considerations were supported by our data showing that education was associated with parity ($P < 0.001$), age at first birth ($P < 0.001$) and use of hormonal contraceptives ($P < 0.01$), tobacco ($P < 0.01$) and alcohol ($P < 0.05$) in the EBBA-I study population.

Reproductive factors may induce permanent changes in the mammary gland epithelium or the surrounding stromal tissue; and the most prominent effects may be related to occurrence and timing of the first pregnancy (Pike et al.1983, Henderson et al. 1988, Naumov et al. 2006, Russo et al. 2008, NBCG). It is possible that breast tissue differentiation can make the breasts more or less susceptible to carcinogenic factors, and the effects may also depend on the underlying genetic susceptibility for breast cancer (Britt et al. 2007). Therefore, it seems conceivable that factors related to age at menarche and timing of first

full-term pregnancy could initiate or inhibit specific types of breast cancer with different aggressiveness (Li et al. 2008).

5.2.3 Ovarian hormones and mammographic density

Our analysis of complete ovarian hormone profiles based on daily measurements in the EBBA-I Study shows that greater percent mammographic density (≥ 28.5%) is associated with significantly higher salivary levels of estradiol and progesterone during the menstrual cycle. Similarly, higher serum concentrations of late follicular and late luteal estradiol and luteal progesterone were also associated with having greater percent mammographic density in our study. The consistency of findings between saliva and serum analyses supports that we have observed true effects. Thus, we hypothesize that hormone concentrations measured in a single blood sample drawn in a timed and specific cycle phase may be useful biomarkers for predicting long-term hormone levels in premenopausal women. Furthermore, we observed a strong dose-response relationship with three to four-fold increased odds of having high percent mammographic density (≥ 28.5%) among women in the upper quartiles of estradiol and progesterone.

Our findings are consistent with previous reports. Weak positive associations between concentrations of follicular serum estradiol (Yong et al. 2009) and luteal serum progesterone (Noh et al. 2006) and percent mammographic density have been observed among premenopausal women. Furthermore, total urinary estrogen metabolites were positively associated with percent mammographic density in premenopausal women (Mascarinec et al. 2012), and a direct association was observed between preovulatory and luteal phase urinary estrone glucuronide and percent mammographic density (Walker et al. 2009). In contrast to our results, the magnitude of the association was considerably reduced after adjustment for BMI; however, these women were mostly parous and older (Walker et al. 2009), and the timing of the mammographic density assessment was not coordinated across the menstrual phase. Others have also observed contradictory findings in which

luteal serum estradiol was unrelated to mammographic density among premenopausal women (Boyd et al. 2002b, Noh et al. 2006).

The suggested effect of both endogenous estrogen and progesterone on mammographic density in premenopausal women in our study is supported by reports from randomized trials including postmenopausal women, showing that combined estrogen plus progesterone use is associated with larger changes in percent mammographic density compared with estrogen use alone (Greendale et al. 2003, McTiernan et al. 2005, McTiernan et al. 2009). These results provide strong support for a causal relation between endogenous ovarian hormone levels and mammographic density.

Furthermore, our results are consistent with the hypothesis that a positive association between circulating free estrogen and progesterone and breast cancer risk may be mediated, in part, by mammographic density. Recently, changes in mammographic density by hormone exposure were observed to be stronger in women who later developed breast cancer (Boyd et al. 2011). Furthermore, breast tumors have been shown to arise predominantly within the radiodense areas of the breast (Pinto Pereira et al. 2011). Thus, mammographic density and levels of endogenous estradiol and progesterone at a given age may together be important markers for breast cancer risk later in life.

Almost 50 % (n = 98) of the EBBA-I women aged 25-35 years had given birth. First full-term pregnancy represents a major event both in relation to breast development (fully developed), mammographic density (less dense), and breast cancer risk (reduced). Thus, analysis stratified by parity is particularly important. In our study, ovarian hormones were more strongly associated with mammographic density among nulliparous women than among parous women. This finding is consistent with an observed association between SNPs on genes involved in the estrogen pathway and mammographic density among premenopausal nulliparous women (Dumas and Diorio 2010), suggesting that the relationship between ovarian hormones and breast density may vary by parity among

premenopausal women. The observed interaction by parity may be explained by the fact that percent mammographic density decreases after first full-term pregnancy (Loehberg et al. 2010). Parity-induced molecular changes in growth factors, cell fate, p53 activation or induction of a specific genomic signature in the breast may be involved (Ginger and Rosen 2003, Balogh et al. 2006). Thus, we hypothesize that relative to the breast tissue of parous women, the breast tissue of nulliparous women may be more susceptible to higher endogenous ovarian hormone concentrations influencing percent mammographic density and breast cancer risk. Accordingly, interventions to reduce hormonal levels may be particularly important in premenopausal years before first full-term pregnancy. When analyzing the associations between ovarian hormones, parity and mammographic density, further stratifications (e.g. by BMI) could have been valuable to identify possible effect modification; however, this was not possible in our study due to the sample size.

We are the first to comprehensively describe the positive association between free and biologically active estrogen and progesterone across an entire menstrual cycle and percent mammographic density among healthy premenopausal women with regular menstrual cycles. A substantial reduction in mortality rates from breast cancer has been observed among Swedish women aged 40-49 years invited to mammography screening (Hellquist et al. 2011). These findings indicate that the targeted age-group for national screening programs should be considered. However, the exposure to radiation during screening should be a part of the discussion together with evaluation of other disadvantages and benefits of including younger healthy women into a mammography screening program (Cancer in Norway 2009). Meanwhile, our findings support the hypothesis that lowering levels of estradiol and progesterone in young women through lifestyle interventions such as aerobic physical activity (Williams et al. 2010, Kossmann et al. 2011) and low-fat diets (Gann et al. 2003, Aubertin-Leheudre et al. 2008) may reduce mammographic density and improve diagnostics and breast cancer risk assessment (Ursin et al. 2001, Ying 2012).

6 Conclusions

In summary, our findings suggest that genetic markers, lifestyle and reproductive factors may influence levels of cycling 17β-estradiol and progesterone in premenopausal women. Moreover, these levels of cycling endogenous ovarian hormones, key factors in breast cancer development, may be associated with premenopausal mammographic density.

More specifically we conclude that:

- The *CYP17* rs2486758 minor allele, and particularly in combination with high levels of metabolic risk factors, is associated with higher levels of daily free and biologically active 17β-estradiol in healthy premenopausal women. This gene-lifestyle interaction needs to be replicated and further evaluated in relation to breast cancer susceptibility in larger populations and among different ethnicities.

- Lifestyle factors including larger waist circumference and previous oral contraceptive use are associated with higher levels of estradiol, particularly among nulliparous women. This suggests that women before first full-term pregnancy may be more susceptible to the effects of these lifestyle factors on cycling estradiol. A shorter 'menarche-to-first birth' time interval is associated with higher levels of daily endogenous 17β-estradiol in a dose-response manner. These findings demonstrate the complexity of the relationship between reproductive factors, lifestyle, fecundity, and ovarian hormone concentrations, with potential implications for breast cancer development.

- Concentrations of 17β-endogenous estradiol and progesterone are strongly positively associated with percent mammographic density in premenopausal women in a dose-response manner. We hypothesize that these patterns may, in part, explain the positive association between mammographic density and breast cancer development. However, more studies are needed to evaluate and confirm the clinical implications of these findings.

7 Implications for further research

The findings in this thesis related to genetic susceptibility, lifestyle, reproductive factors, ovarian hormones and mammographic density suggest several interesting questions to be further explored. The functional significance of *CYP17* rs2486758 genotype with respect to estrogen levels and interactions with metabolic risk factors should be confirmed in larger studies and among different ethnicities. Moreover, all the primary genotyped SNPs in the EBBA-I Study should be studied in relation to sex steroids including androgens in order to further explore associations, patterns and combined effects of susceptibility SNPs, gene-gene interactions and interactions with lifestyle factors possibly underlying the complexity of breast cancer development. Exploring the associations between the genotyped SNPs in the EBBA-I Study with mammographic density may provide further information about biological mechanisms and should include test for interactions with lifestyle factors.

Furthermore, serum based biomarkers in relation to mammographic density may be further explored in relevant study designs and populations in order to improve methods for early breast cancer detection, and for targeting high risk groups for primary and secondary breast cancer prevention. Intervention studies involving physical activity and diets should be designed to examine their effects on mammographic density among healthy women and in breast cancer patients, in order to improve breast cancer prevention.

Follow-up studies of women participating in the EBBA-I Study will provide cohort data that allow prospective studies of important associations, as well as life changes in these women who may have more children, and will be approaching the menopausal transition and the menopause. This will provide several possibilities including data collection from more than one menstrual cycle, and if desirable, with collection from various seasons. Consequently, new information of breast cancer risk factors and mechanisms may be found.

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Paper I

Genetic Polymorphism *CYP17* rs2486758 and Metabolic Risk Factors Predict Daily Salivary 17β-Estradiol Concentration in Healthy Premenopausal Norwegian Women. The EBBA-I Study

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Context: The relationship between low-penetrance genes, metabolic risk factors, and levels of endogenous 17β-estradiol and progesterone, which play a role in breast cancer risk, remains unclear.

Objective: The aim of this study was to determine whether common polymorphisms in *CYP17*, in combination with metabolic risk factors (individually or clustered), alter salivary concentrations of free biologically active 17β-estradiol and progesterone among healthy premenopausal Norwegian women.

Design: Eight single nucleotide polymorphisms in *CYP17* were genotyped in 203 healthy premenopausal women aged 25–35 yr in the Norwegian EBBA-I Study, conducted in 2000–2002. Daily salivary concentrations of 17β-estradiol and progesterone were measured throughout one menstrual cycle. A clustered metabolic score was calculated, including waist circumference, mean arterial pressure, insulin resistance, fasting triglycerides, and total cholesterol/high-density lipoprotein cholesterol ratio. The study hypothesis was tested in multivariable linear regression and generalized estimating equation models.

Results: Women in the upper tertile of clustered metabolic score with the *CYP17* rs2486758 minor allele had daily salivary 17β-estradiol concentrations that were 53% higher than other study women throughout the menstrual cycle ($P < 0.001$). Similarly, women in the upper tertile of total cholesterol/high-density lipoprotein cholesterol ratio, fasting triglycerides, and insulin resistance had 44, 32, and 24% higher daily salivary 17β-estradiol concentrations, respectively (all $P < 0.05$).

Conclusion: The *CYP17* rs2486758 minor allele may predispose to higher 17β-estradiol levels, particularly in premenopausal women with a high clustered metabolic score. Thus, modification of metabolic risk factors may have significant implications for the prevention of breast cancer in women with the minor allele of *CYP17* rs2486758. (***J Clin Endocrinol Metab* 97: E852–E857, 2012)**

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Abbreviations: HDL-C, High-density lipoprotein cholesterol; HOMA, homeostatic model assessment; SNP, single nucleotide polymorphism.

Exposure to ovarian hormones is critical to breast cancer development; the genes that control estrogen and progesterone biosynthesis in the ovaries may contribute to inherent variability in breast cancer susceptibility. The present study focuses on *CYP17*, which is expressed in ovarian theca cells and encodes cytochrome P450 enzymes that control the early steps of endogenous estrogen biosynthesis by converting progesterone into precursors of estrogen.

So far, reports have failed to demonstrate a consistent relationship between *CYP17* genotype and levels of ovarian hormones in premenopausal women (1–4) or breast cancer risk (5, 6). However, mainly rs743572, which is a single nucleotide polymorphism (SNP) located in the promoter region of *CYP17*, has been studied, whereas recent evidence indicates that SNPs in noncoding regions of a gene may effect gene expression. Interestingly, when stratifying by body mass index, Small *et al.* (2) documented an association between *CYP17* rs743572 and serum estradiol concentration among leaner women. Importantly, metabolic risk factors (*i.e.* body fatness, hypertension, dyslipidemia, and insulin resistance) increase levels of ovarian hormones (7–10) and breast cancer risk (5, 6), but little is known about interacting effects of genetic predisposition caused by other common SNPs in *CYP17*.

Based on these previous studies, there is a need for further investigations to address possible modifying effects of metabolic risk factors on the association between common SNPs in *CYP17* and ovarian hormones. Therefore, the main aim of this study was to examine whether eight SNPs in *CYP17*, in combination with higher levels of metabolic risk factors (individually or clustered), are associated with increased daily salivary 17β-estradiol and progesterone concentrations in healthy premenopausal women with regular ovulatory cycles.

Subjects and Methods

Participants and study design

The Norwegian Energy Balance and Breast Cancer Aspects (EBBA-I) Study was conducted in 2000–2002 and included 204 women aged 25–35 yr with regular menstrual cycles (length, 20–40 d) who did not use any daily medication; were not pregnant, lactating, or using steroid contraceptives in the 6 months before recruitment; and had no gynecological or chronic disorders (7). Characteristics including reproductive history and lifestyle factors were collected using questionnaires at the time of recruitment. One woman was excluded due to low call frequency in the genotyping analysis; thus, 203 participants are included in this report. All participants gave written informed consent, and ethical approval was obtained for the study.

Clinical examination and collection of blood samples

All participants underwent clinical examination at the Clinical Research Center, University Hospital of North Norway, Tromsø, Norway, at three scheduled visits during their menstrual cycle. The present report considered measures from the first visit only. Morning blood samples were taken at the first scheduled visit (between d 1 and 5 of the menstrual cycle), after a fasting period that started at 2400 h and included abstaining from smoking and exercise. Details of the EBBA-I Study, anthropometric measurements, and serum lipids, glucose and SHBG assays have been described (7) and are included in the Supplemental Materials and Methods (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Collection and analysis of saliva samples

Participants self-collected daily morning saliva samples at home for one entire menstrual cycle, starting on the first day of bleeding (7, 11).

Salivary 17β-estradiol concentration was assayed for 20 d and progesterone for 14 d of the menstrual cycle, using I-125-based RIA kits (see Supplemental Materials and Methods for details). All cycles were aligned at midcycle based on the identification of the drop in 17β-estradiol concentration (aligned cycle day 0), which provides a reasonable estimate of the day of ovulation (11). Satisfactory identification of the drop in salivary 17β-estradiol concentration could not be made for 14 women. For the remaining 189 women, a "late-follicular" index (mean value aligned cycle days −5 to −1), a "luteal" index (mean value aligned cycle days 0 to + 6), and a "mid-menstrual" index (mean value aligned cycle days −7 to + 6) were calculated. Maximum peak level refers to the highest measured hormone value during the menstrual cycle (aligned cycle day −1).

SNP selection and genotyping

To represent the variability in *CYP17* in Caucasians, eight of its SNP (rs1004467, rs743575, rs4919687, rs3781286, rs3824755, rs10786712, rs743572, and rs2486758) were selected using the Genome Variation Server (LDSelect). Criteria for SNP selection were an r^2 threshold of 0.8 and a minor allele frequency greater than 5%. Genotyping was performed using the Illumina Golden Gate Platform (Illumina Inc., San Diego, CA).

Homo- and heterozygote genotypes of the minor allele were combined into one category, using the homozygote genotype of the major allele as the reference category (dominant model). Five SNPs in *CYP17* were not associated with ovarian hormones, and interactions with metabolic risk factors were not observed in crude analyses. Another two (rs743575, rs4919687) were weakly associated with overall mean salivary 17β-estradiol concentration in crude analyses, but no interactions with metabolic risk factors were observed. Therefore, these seven SNPs were considered less suitable for the main aim of this report and were discarded. One SNP in *CYP17* (rs2486758) was associated with overall mean salivary 17β-estradiol concentrations in crude analyses and was therefore used in further analyses. Further information about SNP selection, as well as details about genotyping and quality control including allele frequencies and a linkage disequilibrium plot, is reported in the Supplemental Materials and Methods, Supplemental Table 1, and Supplemental Fig. 1.

Statistical analyses

Statistical analyses were performed using STATA version SE 11.0 (StataCorp., College Station, TX). The statistical significance level was defined as $P < 0.05$. More details are available in the Supplemental Materials and Methods and Supplemental Table 3.

Descriptive analyses

Student's *t* test and Pearson's χ^2 test were used to compare means and proportions of selected characteristics of the participants by genotype. The homeostatic model assessment (HOMA) score [fasting glucose (mmol/liter) × fasting insulin (μIU/ml)]/22.5 was used as an indicator of insulin resistance. We constructed a clustered metabolic score by summarizing z-scores [(individual raw values − sample mean)/sample SD] of the following variables for each woman: waist circumference, fasting triglycerides, total cholesterol/high-density lipoprotein cholesterol (HDL-C) ratio, HOMA score, and mean arterial pressure [(diastolic blood pressure × 2) + systolic blood pressure]/3. Metabolic score was considered a continuous variable with a mean score of 0, so that lower values corresponded to a more favorable profile.

Analyses of single SNP

Linear regression and generalized estimating equation models were used to evaluate associations between *CYP17* SNP and salivary ovarian hormone concentrations. All hormone values were log-transformed before the statistical analyses. Possible interactions between *CYP17* SNP were evaluated by multiplicative terms in the models with age as the only covariate. Wald χ^2 test statistics was used to assess the associations between *CYP17* rs2486758 genotypes, metabolic risk factors, and daily salivary 17β-estradiol concentrations. The study population was homogeneously Caucasian; thus, ethnicity was not included in the model as a covariate. Participants were stratified by tertiles of metabolic risk factors (individual and clustered).

TABLE 1. Characteristics of the Norwegian EBBA-I Study population by *CYP17* rs2486758 genotypes (n = 203)[a]

	Major allele[b]	Minor allele[b]	*P* value[c]
n	121	82	
Age (yr)	30.7 (30.1, 31.2)	30.8 (30.1, 31.4)	0.85
Age at menarche (yr)	13.0 (12.8, 13.3)	13.3 (12.9, 13.6)	0.25
Cycle length (d)	28.5 (28.0, 29.1)	27.8 (27.0, 28.5)	0.087
Energy intake (kJ/d)	8192 (7853, 8531)	7915 (7494, 8336)	0.31
Alcohol (units/wk)	3.26 (3.03, 3.49)	3.14 (2.85, 3.42)	0.50
Current smokers (%)	23.1	20.7	0.69
Sedentary activity in leisure time (%)	59.4	40.6	0.98
Body composition			
BMI (kg/m^2)	24.2 (23.6, 24.9)	24.5 (23.7, 25.4)	0.59
Waist circumference (cm)	79.0 (77.3, 80.7)	79.9 (77.8, 82.0)	0.54
Clinical measurements			
Systolic blood pressure (mm Hg)	112.5 (110.4, 114.6)	114.4 (112.1, 116.7)	0.23
Diastolic blood pressure (mm Hg)	70.5 (68.9, 72.0)	71.5 (70.0, 73.1)	0.37
Serum concentrations[d]			
Total cholesterol (mmol/liter)	4.48 (4.34, 4.63)	4.39 (4.23, 4.55)	0.41
HDL-C (mmol/liter)	1.55 (1.49, 1.61)	1.53 (1.45, 1.60)	0.64
Total cholesterol/HDL-C ratio	3.00 (2.86, 3.15)	3.00 (2.82, 3.18)	0.97
Triglycerides (mmol/liter)	0.78 (0.70, 0.86)	0.99 (0.64, 1.34)	0.17
Glucose (mmol/liter)	4.97 (4.87, 5.07)	5.09 (4.96, 5.22)	0.15
Insulin (pmol/liter)	85.2 (72.9, 97.4)	84.1 (75.9, 92.3)	0.90
SHBG (nmol/liter)[e]	52.6 (49.1, 56.2)	51.0 (46.7, 55.3)	0.57
Clustered metabolic score[f]	2.07 (1.44, 2.70)	2.91 (1.97, 3.85)	0.13
Saliva concentrations (pmol/liter)[e]			
Overall 17β-estradiol[g]	13.0 (11.8, 14.3)	15.4 (13.7, 17.2)	0.026
Late follicular index 17β-estradiol[h]	16.7 (15.0, 18.6)	18.4 (16.2, 21.0)	0.11
Luteal index 17β-estradiol[i]	13.9 (12.3, 15.6)	16.4 (14.3, 18.8)	0.035
Overall progesterone[g]	90.0 (78.8, 102.8)	101.4 (87.3, 117.7)	0.24

Data are expressed as mean (95% confidence interval) or percentage unless otherwise specified. BMI, Body mass index.

[a] Number may vary due to missing information.

[b] Major allele, homozygote; minor allele, hetero- and homozygote.

[c] Student's *t* test or Pearson's χ^2 test, linear regression, or generalized estimating equation with log-transformed hormones as dependent variable.

[d] Fasting serum measurements at d 1–5 after onset of menstrual cycle.

[e] Data represent age-adjusted geometric means (95% confidence interval).

[f] Estimated from waist circumference, fasting triglycerides, total cholesterol/HDL-C ratio, mean arterial pressure [(diastolic blood pressure × 2) + systolic blood pressure]/3, and HOMA score [fasting glucose (mmol/liter) × fasting insulin (μIU/ml)]/22.5.

[g] Genotypes equally distributed among women with and without identified drop day.

[h] Aligned cycle day −5, −1 (n = 189).

[i] Aligned cycle day 0, +6 (n = 189).

Haplotype analyses of multiple SNP

Haplotype analyses were performed by using SNPStats software (Catalan Institute of Oncology, IDIBELL, Epidemiology and Cancer Registry L'Hospitalet, Barcelona, Spain) (12). The eight selected SNP formed nine common haplotypes (frequency > 0.5%) in our study population. Likelihood ratio test was used to assess the association between the haplotypes and salivary 17β-estradiol concentration.

Results

Analyses of a single SNP

CYP17 rs2486758 genotype and ovarian hormones

Participants with the *CYP17* rs2486758 minor allele had an 18.5% higher overall salivary 17β-estradiol concentration (P = 0.026), and an 18.0% higher luteal index (P = 0.035), compared with women with the homozygote genotype of the major allele (Table 1). Analyses of salivary 17β-estradiol concentration by aligned cycle day are reported in Supplemental Table 2 and Supplemental Fig. 2. *CYP17* rs2486758 genotype was not associated with salivary progesterone and serum SHBG concentrations (Table 1).

Comparison across tertiles of metabolic risk factors: CYP17 rs2486758 genotype and 17β-estradiol

For participants in the upper tertile of metabolic risk factors with the minor allele, we observed an increase in daily salivary 17β-estradiol concentration throughout the menstrual cycle: 24% for HOMA score (P = 0.021), 32% for fasting triglycerides (P = 0.004), 44% for total cholesterol/HDL-C ratio (P = 0.005), and 53% for clustered metabolic score ($P < 0.001$), compared with all other women combined (Fig. 1). *CYP17* rs2486758 was not associated with serum SHBG concentration among women in the upper tertile of the clustered metabolic score (P = 0.19).

Comparison within the upper tertile of metabolic risk factors: CYP17 rs2486758 genotype and 17β-estradiol

For women in the upper tertile of metabolic risk factors, the minor allele was associated with an increase in daily salivary 17β-estradiol concentration of 29% for fasting triglycerides (P = 0.040), 35% for total cholesterol/HDL-C ratio (P = 0.039), and 38% for clustered metabolic score (P = 0.004) compared with women in the upper tertile of metabolic risk factors carrying the major allele (Fig. 1). The increase in peak salivary 17β-estradiol concentration was 36% (P = 0.024) for clustered metabolic score (Fig. 1F).

Haplotype analyses

One specific haplotype was associated with increased salivary 17β-estradiol concentration (P = 0.0045). There was no significant difference in salivary 17β-estradiol concentration between haplotypes (global haplotype association P value = 0.65) (Table 2).

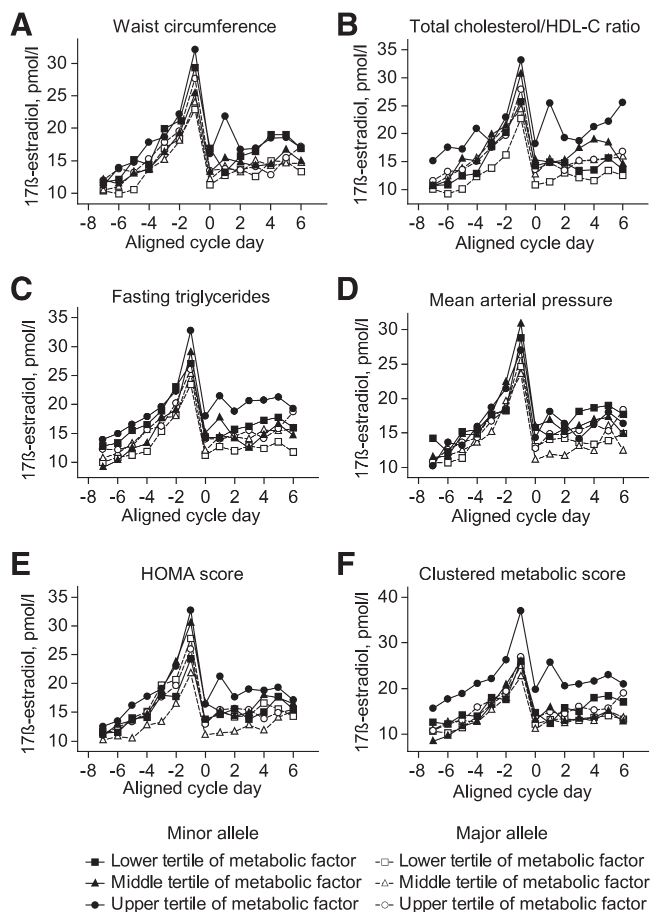

FIG. 1. Daily salivary 17β-estradiol concentrations (geometric means) in midmenstrual cycle (aligned cycle day, −7, 6) for women categorized by *CYP17* rs2486758 genotype and tertiles of metabolic risk factors. Major allele, homozygote; minor allele, hetero- and homozygote. A, Waist circumference. Major allele and lower tertile (n = 32), middle tertile (n = 41), upper tertile (n = 38); minor allele and lower tertile (n = 23), middle tertile (n = 27), upper tertile (n = 28). B, Total cholesterol/HDL-C ratio. Major allele and lower tertile (n = 36), middle tertile (n = 34), upper tertile (n = 38); minor allele and lower tertile (n = 27), middle tertile (n = 27), upper tertile (n = 22). C, Fasting triglycerides. Major allele and lower tertile (n = 37), middle tertile (n = 38), upper tertile (n = 34); minor allele and lower tertile (n = 27), middle tertile (n = 24), upper tertile (n = 25). D, Mean arterial pressure calculated by the equation: [(diastolic blood pressure × 2) + systolic blood pressure]/3. Major allele and lower tertile (n = 41), middle tertile (n = 34), upper tertile (n = 36); minor allele and lower tertile (n = 21), middle tertile (n = 28), upper tertile (n = 29). E, HOMA score calculated by the equation: [fasting glucose (mmol/liter) × fasting insulin (μIU/ml)]/22.5. Major allele and lower tertile (n = 39), middle tertile (n = 41), upper tertile (n = 29); minor allele and lower tertile (n = 22), middle tertile (n = 21), upper tertile (n = 33). F, Clustered metabolic score estimated from: waist circumference, fasting triglycerides, total cholesterol/HDL-C ratio, blood pressure as per the equation above, and HOMA score as per the equation above. Major allele and lower tertile (n = 43), middle tertile (n = 31), upper tertile (n = 34); minor allele and lower tertile (n = 22), middle tertile (n = 27), upper tertile (n = 27).

Discussion

To our knowledge, this is the first study to evaluate a set of SNPs, including *CYP17* rs2486758, in relation to ovarian

TABLE 2. Haplotype association with salivary 17β-estradiol concentrations for the eight selected SNP in *CYP17* in the Norwegian EBBA-I Study (n = 203)

		17β-estradiol		
Haplotype[a]	Frequency	Difference	95% CI	*P* value
AAAGCGAA	0.28	0.00	Ref.	
ACGGCGAA	0.19	−0.06	−0.23, 0.12	0.53
AAAACAGA	0.16	0.03	−0.14, 0.21	0.73
AAAGCGAG	0.16	0.25	0.08, 0.43	0.0045
AAAACAGG	0.06	−0.17	−0.47, 0.13	0.26
GAAAGAGA	0.05	0.06	−0.18, 0.31	0.62
ACGACAGA	0.05	−0.04	−0.3, 0.23	0.79
GCGAGAGA	0.03	0.19	−0.15, 0.53	0.28
GAAAGAGG	0.02	0.1	−0.33, 0.53	0.65

Test of equality between haplotypes (global haplotype association), *P* value = 0.65.

[a] Haplotype order, rs1004467 (A>G), rs743575 (A>C), rs4919687 (G>A), rs3781286 (G>A), rs3824755 (C>G), rs10786712 (G>A), rs743572 (A>G), and rs2486758(A>G).

hormone concentrations and metabolic risk factors. We observed a novel association between the *CYP17* rs2486758 minor allele and elevated daily levels of free biologically active estradiol among premenopausal Norwegian women in the upper tertile of metabolic risk factors.

Some previous studies have reported increased levels of salivary and serum estradiol among healthy premenopausal women with the hetero- and homozygote genotype of the *CYP17* rs743572 minor allele (1–3), whereas others reported inconsistent results (4), as has also been the case with serum progesterone (1, 4).

CYP17 rs2486758 is localized in the intergenic section near the 5′ of *CYP17*, and approximately 40% of trait-associated SNPs have been found in intergenic regions (13). Based on current understanding in this field, we can predict that the *CYP17* rs2486758 minor allele may increase *CYP17* expression by effecting gene splicing, transcription factor binding, or the sequence of noncoding RNA (13). Changes in the feedback sensitivity and adjustment of the estradiol setpoint driven by *CYP17* could be a possible explanation for the observed increase in circulating levels of estradiol, and this will have to be tested in further studies.

In the present analysis, *CYP17* rs2486758 was not associated with metabolic risk factors and levels of SHBG. Thus, our data support a true gene-environment interaction, in which only women with the *CYP17* rs2486758 minor allele are susceptible to the possible estrogen-enhancing effects of a high clustered metabolic score. A similar interaction was observed between *CYP17* rs743572 and body mass index in a study of serum estradiol (2). Nutrition and physiological stress have also been reported to change gene expression through epigenetic mechanisms, and there is indirect evidence of epigenetic regulation of *CYP17* from *in vitro* studies (14). *CYP17* expression in the ovaries depends on complex interactions between multiple molecular pathways (15). Moreover, differential tissue-specific regulation of *CYP17* in the ovary and adipose tissue (16) may also have contributed to the interactions observed in our study.

Physiological studies have shown that nutritional status (*i.e.* overweight) and energy metabolism may regulate estrogen levels through different mechanisms; increased energy availability up-regulates ovarian function and hormone secretion in premenopausal years (17). Furthermore, estrogens are formed from androgens in adipose tissue, and adiposity-associated insulin resistance causes reduction in the hepatic production of SHBG (18).

The observed interaction between *CYP17* and metabolic risk factors in the present study may be particularly relevant for breast cancer. Indeed, because the disease seems to cluster in a subset of the female population (19), genetic variation and interactions with lifestyle-related factors may contribute to breast cancer risk, but further studies are needed.

In a larger study, no association between *CYP17* rs2486758 and breast cancer risk was observed (20). On the contrary, an increased breast cancer risk has been linked to *CYP17* rs743572 minor allele carriers (21). Nevertheless, several studies have reported conflicting results and questioned breast cancer susceptibility related to *CYP17* genotypes (6).

Our study suggests that genetically susceptible women with the *CYP17* rs2486758 minor allele may benefit from the modification of metabolic risk factors for the prevention of breast cancer and other estrogen-related diseases (*i.e.* osteoporosis, cardiovascular disease). These are important public health perspectives considering the worldwide increase in the prevalence of obesity, type 2 diabetes, and metabolic syndrome. However, our findings need to be replicated by others and in populations of different ethnicities. Also, studies of breast cancer and other disease outcomes are needed to clarify whether this new knowledge should be incorporated in health care delivery.

Acknowledgments

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Disclosure Summary: There were no conflicts of interest.

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Supplemental data

Materials and methods

Clinical parameters

All clinical measurements were conducted by two trained nurses at the Clinical Research Center, University Hospital of North Norway (UNN), Tromsø, Norway (1). Each participant came to the UNN three times during their menstrual cycle for clinical examination, between days 1–5, days 7–12 and days 21–25. The first visit was on the first day possible after the onset of menstrual bleeding, and was the only visit taken into account in the present report. During this first visit anthropometric measurements were taken with participants wearing light clothing and no footwear. Height was measured to the nearest 0.5 cm, and weight to the nearest 0.1 kg on an electronic scale. Body mass index was measured in kg/m^2. Waist circumference (cm) was measured 2.5 cm above the umbilicus. Blood pressure was measured three times subsequent to a 5-minute resting period in a sitting position, and the mean of the final two measurements was used in the analysis.

Collection and analysis of blood samples

Morning blood samples were taken at each of the three scheduled visits, after a fasting period that started at midnight and included abstaining from smoking and exercise. The present report considered data from the first visit only. Glucose, sex- hormone-binding globulin (SHBG) and lipid levels were measured in fresh serum at the Department of Clinical Chemistry, UNN (1). Serum glucose was measured enzymatically by the hexokinase method. SHBG was measured by an immunometric method (Diagnostic Products Corporation (DPC), Bierman GmbH, Bad Nauheim, Germany). Fasting triglycerides were assayed by enzymatic hydrolysis with lipase. Total cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. High-density lipoprotein cholesterol (HDL-C) was quantified by direct assay using PEG-modified enzymes and dextran sulfate. Insulin was measured at the Hormone Laboratory, Aker University Hospital, Oslo, Norway, in sera stored at -70°C until analysis by radioimmunoassay (RIA) (Linco Research Inc., St. Charles, Missouri, USA).

Collection and analysis of saliva samples

Participants self-collected daily morning saliva samples at home for one entire menstrual cycle starting on the first day of bleeding (1, 4). Hormone assays were run in the Reproductive Ecology Laboratory, Harvard University, Cambridge, Massachusettes, USA.

Salivary 17β-estradiol concentration was assayed for 20 days (reverse cycle days −5 to −24; with -1 implicating the last day of the menstrual cycle) and progesterone for 14 days (reverse cycle days −1 to −14) of the menstrual cycle using I-125-based RIA kits (Diagnostic Systems Laboratories, Webster, TX, USA). All samples were run in duplicate, and all samples from a single participant were run together in the same assay, with women randomly assigned to assay batches. The sensitivity of the 17β-estradiol assay was 4 pmol/l. Average intra-assay variability was 9%, and inter-assay variability ranged from 23% for low pools to 13% for high pools. For progesterone, the sensitivity of the essay was 13 pmol/l. Average intra-assay variability was 10%, inter-assay variability ranged from 19% for low pools to 12% for high pools.

Prior to statistical analysis of daily ovarian hormone levels, the cycles of the participants were aligned at mid-cycle following published methods (4). Alignment was based on the identification of the mid-cycle drop in salivary 17β-estradiol concentration (aligned cycle day 0), which provides a reasonable estimate of the day of ovulation. Satisfactory identification of the mid-cycle drop in salivary 17β-estradiol concentration could not be made for 14 women. For the remaining 189 women with aligned cycles, a "late-follicular" index (mean value aligned cycle days -5 to -1), a "luteal" index (mean value aligned cycle days 0 to + 6), a "Mid-menstrual" index (mean value aligned cycle days -7 to + 6) were calculated. Maximum peak level refers to the highest measured hormone value during the menstrual cycle (aligned cycle day -1). Genotypes were equally distributed among women with and without an identified drop day.

SNP selection and genotyping

DNA was extracted from whole blood using MagAttract DNA Blood Mini M48 kit (Qiagen, Oslo, Norway) by the Department of Medical Genetics, UNN. To represent the variability of *CYP17* in Caucasians, eight single nucleotide polymorphisms (SNPs) (rs1004467, rs743575, rs4919687, rs3781286, rs3824755, rs10786712, rs743572, rs2486758) were selected using the Genome Variation Server (LD select) (2-3). The SNPs were selected at an r^2 threshold of 0.8 and a minor allele frequency of > 5%. TagSNP coverage extends 2 kb upstream and 1kb downstream of the gene. Genotyping was performed at the Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, using the Illumina Golden Gate platform. Included in the assay were 22 blinded replicates, and genotype control for the Caucasian population (NA07034). One sample failed, with a call frequency < 85% and none of the selected SNPs in *CYP17* were monomorphic

or significantly out of the Hardy-Weinberg Equilibrium. Thus, 203 women were included in the present study.

The eight selected SNPs in *CYP17* were organized into four groups, or bins according to r^2, with one highly correlated tagSNP in each bin to ensure the genetic diversity of that bin in the statistical analysis. One SNP from each bin (rs743575, rs3824755, rs743572 and rs2486758) was then used in the crude analysis. Homo- and heterozygote genotypes of the minor allele were combined into one category using the homozygote genotype of the major allele as the reference category (dominant model).

Ethical considerations

All participating women signed an informed consent form and the study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Statistical analysis

All hormone values were log transformed prior to regression analysis and back-transformed for presentation (geometric means and 95% confidence intervals). Inclusion of height, the only covariate associated with genotype, and body mass index in the models did not change the regression coefficients of genotypes to any meaningful extent.

Based on metabolic scores associated with physical activity (5, 6) and breast cancer risk (7) in Norwegian women, and the definition of metabolic syndrome by the International Diabetes Federation, which includes waist circumference as the primary measure of central obesity (8), we constructed a clustered metabolic score by summarizing z-scores [(individual raw values − sample mean) / sample standard deviation] of waist circumference, fasting triglycerides, total cholesterol/HDL-C ratio, homeostatic model assessement (HOMA) score (9), and mean arterial pressure [(2 diastolic blood pressure + 1 systolic blood pressure) / 3]. HOMA score and fasting triglycerides were log transformed prior to the calculation of z-scores. This metabolic score was considered as a continuous variable with a mean of 0, so that lower values corresponded to a more favorable profile. Exploratory factor analysis was used to describe the correlations among the variables in the clustered metabolic score, and to determine the number of factors describing the covariance structure (10). Kaiser's criterion (eigenvalue > 1) and Cattell's scree plot were used for factor extraction. The exploratory factor analysis indicated a satisfactory loading (>

0.400) for all variables except fasting triglycerides (< 0.400). Fasting triglycerides were nevertheless kept in the clustered metabolic score because of biological plausibility (Table III).

Generalized estimating equation models, adjusted for age, with Wald chi-square test statistics were used to assess the associations between *CYP17* rs2486758 genotypes and daily salivary 17β-estradiol concentrations.

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Supplemental Table I. Allele frequencies and distribution of selected SNPs in *CYP17*. The Norwegian EBBA Study (n=203)

SNP	Location (bp)	SNP Position[a]	Alleles[b]	MAF	HWE[c]	AA[d]		Aa[d]		aa[d]	
						n	(%)	n	(%)	n	(%)
rs1004467	104484497	Intron_3	A>G	0.097	0.209	168	(83)	31	(15)	4	(2)
rs743575	104584896	Intron_2	A>C	0.27	0.910	108	(53)	81	(40)	14	(7)
rs4919687	104585238	Intron _1	G>A	0.27	0.986	108	(53)	81	(40)	14	(7)
rs3781286	104585709	Intron_1	G>A	0.37	0.236	86	(42)	86	(42)	31	(16)
rs3824755	104585839	Intron_1	C>G	0.097	0.209	168	(83)	31	(15)	4	(2)
rs10786712	104586386	Intron_1	G>A	0.37	0.236	86	(42)	86	(42)	31	(16)
rs743572	104587142	5' UTR	A>G	0.37	0.236	86	(42)	86	(42)	31	(16)
rs2486758	104587470	Intergenic (GVS) 5' near gene	A>G	0.24	0.221	121	(60)	67	(33)	15	(7)

SNP, single nucleotide polymorphism; MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; A, major allele; a, minor allele.
[a]According to information on dbnSNP home-page: http://www.ncbi.nlm.nih.gov/projects/SNP/
[b]Most frequent to least frequent allele.
[c]p-value for HWE (cut off 0.0010).
[d]Distribution in EBBA study population.

Supplemental Table II. Daily salivary 17β-estradiol concentrations by cycle day (geometric means, 95% confidence intervals, CI) for women categorized by *CYP17* rs2486758 genotypes: The Norwegian EBBA-I Study (n = 189)

Hormonal index (pmol/liter)[c]	Major allele[a] (n = 111) Mean (95% CI)	Minor allele[a] (n = 78) Mean (95% CI)	Diff (%)	P value[b]
Mid-menstrual[d]	14.78 (13.09,16.68)	16.46 (14.31,18.95)	14	0.077
Luteal[e]	13.85 (12.33,15.56)	16.38 (14.28,18.79)	19	0.035
Maximum peak level[f]	24.80 (24.41,27.44)	28.82 (25.55,32.50)	16	0.061

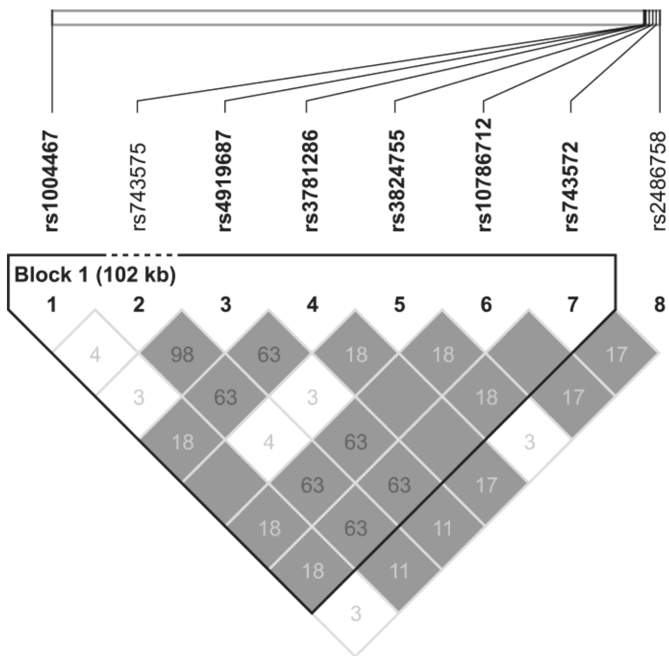
[a]Major allele: homozygote, minor allele: hetero- and homozygote.
[b]Wald chi-square test.
[c]Age-adjusted geometric means of salivary 17β-estradiol concentration for a given number of aligned cycle days.
[d]Aligned cycle day -7,+ 6.
[e]Aligned cycle day 0,+ 6.
[f]Aligned cycle day -1.

Supplemental Table III. Relationships among selected risk factors in the clustered metabolic score[a]. The Norwegian EBBA-I Study (n=203)[b]

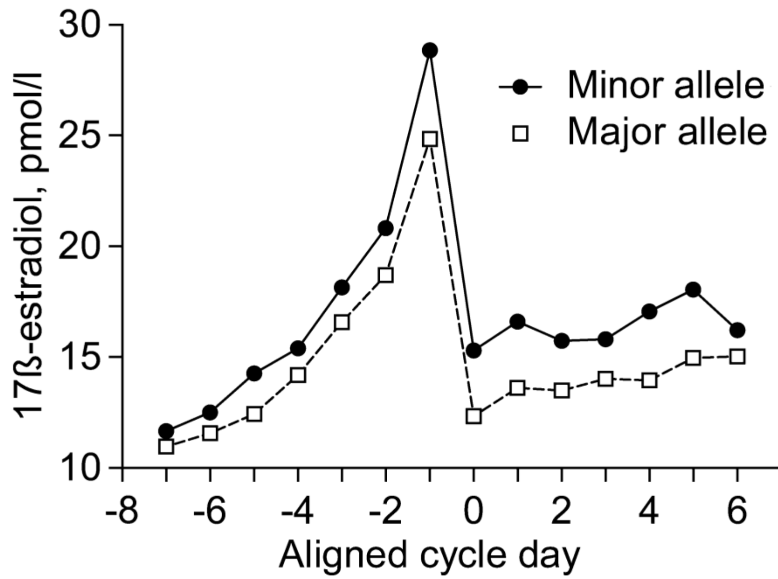
Variable, z-score[c]	Factor loadings[d]	Uniqueness[e]
Waist circumference	0.834	0.304
Fasting triglycerides	0.332	0.890
HOMA score	0.603	0.637
Mean arterial pressure	0.547	0.701
Total cholesterol/HDL-C ratio	0.752	0.435

HDL-C, high-density lipoprotein-cholesterol; HOMA, homeostatic model assessement.

[a]Estimated from sum of z-scores in each women for: waist circumference (cm), total cholesterol/HDL-C ratio, fasting triglycerides mmol/l, mean arterial pressure calculated by the equation: [2 diastolic blood pressure (mmHg) + 1 systolic pressure (mmHg) / 3], and HOMA score given by the equation: [fasting glucose (mmol/l) x fasting insulin (μIU/ml)] / 22.5.
[b]Numbers may vary due to missing information.
[c]z-score calculation; sample mean subtracted from individual score/raw score, divided by the sample standard deviation.
[d]Factor 1 from principal component factor analysis; represents weights for variables and correlation between the variables and the factor.
[e]Proportion of the common variance of the variable not associated with the factor.



Supplemental Figure 1. Linkage disequilibrium structure among the eight selected single nucleotide polymorphisms of *CYP17* (11).



Supplemental Figure 2. Daily salivary 17β-estradiol levels by cycle day (geometric means) in mid-menstrual cycle (aligned cycle day -7, + 6) for women categorized by *CYP17* rs2486758 genotypes; major allele (homozygotes) (n=111) and minor allele (homo- and heterozygotes) (n=78).

Paper II

Human Reproduction, Vol.26, No.6 pp. 1519–1529, 2011
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human reproduction **ORIGINAL ARTICLE** ***Reproductive epidemiology***

Ovarian hormones and reproductive risk factors for breast cancer in premenopausal women: the Norwegian EBBA-I study

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BACKGROUND: Ovarian hormones, parity and length of 'menarche-to-first birth' time interval are known risk factors for breast cancer, yet the associations between 17β-estradiol, progesterone and these reproductive factors remain unclear.

METHODS: A total of 204 women (25–35 years) who participated in the Norwegian EBBA-I study collected daily saliva samples for one complete menstrual cycle, and filled in a reproductive history questionnaire. Anthropometry was measured and saliva samples were analyzed for ovarian hormones. Associations between parity, the interval and ovarian hormones, and effects of hormone-related lifestyle factors were studied in linear regression models.

RESULTS: Mean age was 30.7 years, and age of menarche 13.1 years. Parous women had on average 1.9 births, and age at first birth was 24.5 years. No association was observed between parity and ovarian steroids. In nulliparous women, higher waist circumference (≥77.75 cm) and longer oral contraceptive (OC) use (≥3 years) were associated with higher levels of 17β-estradiol. Short (<10 years) versus long (>13.5 years) 'menarche-to-first birth' interval was associated with higher overall mean ($P_{trend} = 0.029$), 47% higher maximum peak and 30% higher mid-cycle levels of 17β-estradiol. We observed a 2.6% decrease in overall mean salivary 17β-estradiol with each 1-year increase in the interval.

CONCLUSIONS: Nulliparous women may be more susceptible to lifestyle factors, abdominal overweight and past OC use, influencing metabolic and hormonal profiles and thus breast cancer risk. Short time between 'menarche-to-first birth' is linked to higher ovarian hormone levels among regularly cycling women, suggesting that timing of first birth is related to fecundity.

Key words: 17β-estradiol / progesterone / menarche / age at first birth / parity

Introduction

Ovarian function plays a fundamental role in female fecundity and fertility (Lipson and Ellison, 1996), and ovarian hormones are major risk factors for breast cancer initiation and progression (Jasienska and Thune, 2001; Endogenous Hormones and Breast Cancer Collaborative Group, 2002; Eliassen *et al.*, 2006; McTiernan *et al.*, 2006). Furthermore, it is well established that early age at menarche, late age at first birth and low parity increases breast cancer risk, and it is generally thought that reproductive events and their timing may influence breast cancer risk through their effects on differentiation of breast tissue and on hormonal and immunological profiles (Verkasalo *et al.*, 2001; Ma *et al.*, 2006; Li *et al.*, 2008). Given the large changes in both the timing of sexual maturation and childbearing pattern, and the rise in breast cancer incidence worldwide (Kaplowitz, 2006; World Cancer Research Fund/American Institute for Cancer Research, 2007), defining the relationship between

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reproductive history and levels of circulating estradiol and progesterone is of particular importance.

Younger age at menarche is associated with higher cumulative exposure to ovarian hormones throughout life (Apter et al., 1989; Bernstein et al., 1991; Emaus et al., 2008a). Both early age at menarche and delayed first full-term birth translate into a longer 'menarche-to-first birth' time interval, which is recognized as a susceptible period for breast cancer development as the undifferentiated breast tissue is exposed to mitogenic estrogen and progesterone (Russo et al., 1982; Li et al., 2008). Moreover, overweight and obesity are established risk factors for breast cancer among postmenopausal women (Ballard Barbash et al., 2006), and girls with excessive body fat tend to experience early age at menarche (Emaus et al., 2008a) and reduced fecundity (Crosignani et al., 2003).

To our knowledge, data regarding levels of ovarian hormones throughout a menstrual cycle in relation to parity and timing of first birth are very limited. Our previous studies show that 17β-estradiol profiles are associated with age at menarche (Emaus et al., 2008a), body composition from birth to adult life (Jasienska et al., 2006a; Finstad et al., 2009a), metabolic profile in adult life (Furberg et al., 2005; Emaus et al., 2008b) and energy balance throughout the menstrual cycle (Ziomkiewicz et al., 2008). These associations point to further studies of ovarian hormones in relation to both fecundity and fertility, and reproductive risk factors for breast cancer.

Thus, we chose to study the variation in the primary endogenous ovarian hormones in premenopausal years, 17β-estradiol and progesterone. A unique aspect of this study is the daily assessments of salivary 17β-estradiol and progesterone, which represent the free, unbound, biologically active fraction of these hormones (Ellison and Lipson, 1999). Therefore, the main aim of the present study was to elucidate whether daily levels of free and biologically active 17β-estradiol and progesterone throughout an entire menstrual cycle are associated with parity and the 'menarche-to-first birth' time interval.

Materials and Methods

Participants and study design

In the Norwegian EBBA-I study (2000–2002), women aged 25–35 years and living in the municipalities of Tromsø and Balsfjord were recruited by local announcements in media and public meeting places (Furberg et al., 2005). Among those who volunteered to participate, 214 women met the inclusion criteria (age: 25–35 years, self-reported regular menstruation, normal cycle length within the previous 3 months, no use of steroid contraceptives, no pregnancy or lactation over the previous 6 months, no history of gynecological disorder and no chronic disorders, e.g. diabetes and hypo-/hyperthyroidism). Suitable respondents were subsequently enrolled in the study, and a total of 204 healthy women completed the study.

Questionnaires and interview

We used questionnaires to collect information including age at menarche, reproductive history, marital status, education, physical activity, previous use of hormonal contraceptives, smoking and alcohol consumption. Data from a 7-day pre-coded food diary were used to estimate daily energy intake (Furberg et al., 2005; Lillegaard et al., 2005). All questionnaires were checked for inconsistencies, and interview by one trained nurse was performed. Recall and memory-probing aids including a lifetime calendar and a list of examples of milestones, were used to date the reproductive history events (Furberg et al., 2005; Emaus et al., 2008a).

Clinical parameters

All clinical procedures and measurements were conducted by trained nurses at the Clinical Research Center, University Hospital of North Norway (UNN), Tromsø. Each study participant came to the research center three times for clinical examination: first visit (Days 1–5 of the menstrual cycle), second visit (Days 7–12) and third visit (Days 21–25). The first visit was conducted on the first day possible after the onset of menstrual bleeding. Anthropometric measurements were taken with participants wearing light clothing and no footwear (Furberg et al., 2005; Finstad et al., 2009b). Body height was measured to the nearest 0.5 cm, and body weight to the nearest 0.1 kg on an electronic scale. BMI (kg/m^2) was used to estimate relative weight. Waist circumference (WC, cm) was measured in a horizontal line 2.5 cm above the umbilicus, and hip circumference (HC, cm) was measured at the largest circumference of the hip (Finstad et al., 2009a). WC and HC (measured to the nearest 0.5 cm) were used to calculate Waist-to-Hip Ratio (WHR = WC/HC). Blood pressure (BP) was measured three times (PROPAQ 104), with the participants sitting in a resting position, and the mean of the final two measurements was used in the analysis.

Saliva hormone samples and analysis

Women collected daily morning saliva samples at home for one entire menstrual cycle starting on the first day of bleeding. Previously established collection protocols (Lipson and Ellison, 1996) were modified and developed for use (Furberg et al., 2005). Hormone assays were run in the Reproductive Ecology Laboratory at Harvard University, USA.

In each cycle, 17β-estradiol was assayed for 20 days (reverse cycle days: −5 to −24) and progesterone was assayed for 14 days (reverse cycle days: −1 to −14), and all values were used in calculation of overall mean hormone concentrations for all participants. Salivary 17β-estradiol and progesterone measurements were made using I-125 based radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories, Webster, TX, USA) with published modifications to the manufacturer's protocols (Furberg et al., 2005). All samples were run in duplicate, and all samples from an individual were run in the same assay, with women randomly assigned to assays.

Saliva pools characterized by high or low hormone values (appropriate to the range of each steroid) were run in each assay. The sensitivity of the 17β-estradiol assay (lowest value measurable by assay) was 4 pmol/l. Average intra-assay variability was 9% and inter-assay variability ranged from 23% for low pools to 13% for high pools. For progesterone, the sensitivity of the assay was 13 pmol/l. Average intra-assay variability was 10%, and inter-assay variability ranged from 19% for low pools to 12% for high pools.

Before statistical analysis of daily hormonal levels, all cycles were aligned at mid-cycle following published methods (Lipson and Ellison, 1996). Alignment was based on the identification of the mid-cycle 17β-estradiol drop (aligned cycle Day 0), which provides a reasonable estimate of the day of ovulation. Identification of the mid-cycle 17β-estradiol drop could not be made for 14 women, and they were not included in subsequent analyses. For the remaining 99 nulliparous and 91 parous women with aligned cycles, the following follicular and luteal hormonal indices were calculated: 'mid-follicular' (defined as the average of values for aligned cycle Days −10 to −6); 'late-follicular' (defined as the average of values for aligned cycle Days −5 to −1); 'luteal' (defined as the average of values for aligned cycle Days +2 to +9); 'mid-menstrual' (defined as the average of values for aligned cycle Days −7 to +6); 'mid-cycle' (defined as the average of values for aligned cycle Days −4 to +2). Maximum peak

level refers to the highest measured hormone value during the mid-menstrual index.

The 17β-estradiol levels in saliva represent the free, unbound, biologically active fraction of the circulating steroid only, rather than the levels of both free and protein-bound 17β-estradiol as in serum [i.e. bound to sex-hormone-binding globulin (SHBG) and albumin] (Ellison and Lipson, 1999). Furthermore, as saliva can readily be collected from individuals on many occasions, it is possible to compare 17β-estradiol levels across entire menstrual cycles among different women, rather than relying on one or a few timed blood samples (Jasienska *et al*., 2006b).

Serum lipid samples and analysis

Fasting serum blood samples were drawn from an antecubital vein in the morning on each of the three visits. Lipids were measured at the Department of Clinical Chemistry, University Hospital of North Norway using fresh sera from the first visit (Furberg *et al*., 2005). Serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. High-density lipoprotein cholesterol (HDL-C) was quantified by a direct assay using polyethylene glycol modified enzymes and dextran sulfate.

Ethical considerations

All participating women signed an informed consent form, and the study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Statistical analysis

We used multivariable linear regression models to study whether parity and the timing of births in relation to age at menarche were associated with levels of salivary 17β-estradiol and progesterone (STATA version SE 11.0). All hormone data were log transformed prior to the linear regression analyses: for presentation, all hormone values were transformed back to the original scale (geometric Means and 95% Confidence Intervals).

To study the associations between parity and 17β-estradiol and progesterone, nulliparous women were compared with parous women and potentially confounding factors were taken into account on the basis of biological plausibility. Parity was included as a dichotomous (nulliparous versus parous), and a continuous (number of full-term childbirths) predictor variable in separate linear regression models. The following potentially confounding factors were assessed on the basis of biological plausibility: BMI, cycle length, age at menarche, smoking, alcohol, physical activity and previous use of oral contraceptives (OCs). However, only minor changes in the regression coefficient of parity were observed for each of these covariates in the models. Thus, age was the only covariate included in the final models. We elucidated possible effect modification and thresholds for variables of importance for the association between parity and ovarian hormones. Thus, we stratified by number of children (0, 1–2, ≥3), body composition: BMI (overweight, ≥25 kg/m^2) and WC (Median, ≥77.75 cm), and previous OC use (Median ≥ 3 years). Generalized estimating equation (GEE) regression models were used to assess the association between daily salivary 17β-estradiol concentrations and parity groups.

The parous women were further categorized into tertiles of the interval between age at menarche and age at first full-term birth: <10 years, 10–13.5 years and >13.5 years. The tertile groups were compared according to characteristics associated with breast cancer risk and/or fecundity. The 'menarche-to-first birth' time interval was included as a continuous and categorical predictor in different regression models. The same potentially confounding factors were evaluated as in the parity models, and only minor changes in the regression coefficient of the 'menarche-to-first birth' interval were observed. Thus, age was the only covariate included in the final models. Linear and logistic regression analyses were used to assess linear trends over tertiles of the 'menarche-to-first birth' interval.

To study whether variation in age at participation and BMI modified the associations between ovarian hormones and the 'menarche-to-first birth' interval (tertiles), age and BMI were dichotomized by median split (33 years) and the cut off for overweight (≥25 kg/m^2), respectively. Possible two-way interactions between the 'menarche-to-first birth' interval and age, BMI, WC, and OC use were assessed in separate models. GEE regression models were used to assess the associations between daily salivary 17β-estradiol concentrations and groups of women in different 'menarche-to-first birth' intervals.

Area under the curve (AUC) for the time–salivary hormone concentration curves was calculated using the trapezium rule (Matthews *et al*., 1990). Measurements of 17β-estradiol for the 14 mid-menstrual days (aligned cycle Days −7 to +6), the 10 late follicular days (aligned cycle Days −10 to −1), the 7 mid-cycle days (aligned cycle Day −4 to +2) and of progesterone for the 8 luteal days (aligned cycle Days +2 to +9) were used in AUC calculations. Linear interpolation (i.e. the mean of the days immediately prior and following) was used to assign a value to days with missing values. If the missing value appeared at the end of the interval, the value from the day next to the missing value was used. One cycle for 17β-estradiol and two cycles for progesterone were excluded from calculations due to two or more missing days at one of the ends of the interval. Among the 189 women included in the AUC analysis, the average number of missing values per cycle was 0.6 days for 17β-estradiol in both parous and nulliparous women and 0.5 days (parous women) and 0.3 days (nulliparous women) for progesterone; both hormones had a range of 0–4 missing days per cycle. Linear regression was used to assess the differences in AUC between parous groups and tertiles of the 'menarche-to-first birth' interval.

Results

Parity and hormonal levels

The average age of nulliparous women ($n = 106$) was 29.2 years (range: 25.0–35.3) and the average age of parous women ($n = 98$) was 32.4 years (range: 24.9–35.9). Mean reported age at menarche was 13.2 years (range: 10.5–19.5) for nulliparous and 13.1 years (range: 9.20–17.0) for parous women (Table I). Mean age at first full-term pregnancy was 24.5 years (range: 16.0–32.0), and parous women had on average 1.9 children (range: 1–5). Compared with nulliparous women, parous women were older ($P < 0.001$), with a higher BMI ($P = 0.012$), larger WC ($P < 0.001$), lower HDL-C ($P = 0.049$) and had lower alcohol consumption ($P < 0.001$) (Table I).

There was no difference in overall mean salivary 17β-estradiol level ($P = 0.31$) or overall mean salivary progesterone level ($P = 0.91$) between nulliparous and parous women (Table II) or between women who had given birth to one child compared with women who had given birth to multiple children (results not presented in table). We observed no difference in average daily level of salivary 17β-estradiol throughout the entire menstrual cycle among three parity groups (nulliparous, 1–2 children, 3–5 children; $P = 0.57$, adjusted for age; Fig. 1A).

When subjects were stratified by BMI and parity, there was a difference in average salivary 17β-estradiol levels throughout the entire

Table I Characteristics of the study population according to parity. The Norwegian EBBA-I study (n = 204)[a].

Characteristic	Parous (n = 98[a])		Nulliparous (n = 106[a])		P-value[b]
	Mean	(SD)	Mean	(SD)	
Age, years	32.4	(2.56)	29.2	(2.71)	<0.001
Education, total years	15.1	(3.09)	17.0	(2.63)	<0.001
Partnership, total years	8.16	(5.49)	2.95	(3.69)	<0.001
Body composition[c]					
Height, cm	167.1	(6.51)	166.6	(6.51)	0.58
BMI, kg/m^2	25.1	(3.83)	23.7	(3.58)	0.010
WC, cm	81.9	(10.1)	77.3	(8.97)	0.001
Waist to hip ratio	0.79	(0.06)	0.76	(0.06)	<0.001
Serum lipids, fasting					
Total cholesterol, mmol/l	4.51	(0.79)	4.39	(0.77)	0.25
HDL cholesterol, mmol/l	1.49	(0.34)	1.58	(0.32)	0.049
Triglycerides, mmol/l	0.87	(1.08)	0.87	(1.01)	0.99
Clinical measurements					
Systolic blood pressure (mmHg)	113.3	(12.1)	113.3	(10.3)	0.99
Diastolic blood pressure (mmHg)	71.0	(8.24)	70.8	(7.83)	0.87
Reproductive history					
Age at Menarche, years	13.1	(1.42)	13.2	(1.32)	0.67
Cycle length, days	27.8	(2.96)	28.6	(3.31)	0.61
Number of children	1.88	(0.88)			
Previous use of oral contraceptives, years	4.44	(3.85)	3.24	(3.46)	0.020
Time since last use of oral contraceptives, years	7.13	(4.47)	3.84	(3.47)	<0.001
Energy intake, kJ/day	8007	(1991)	8173	(1917)	0.53
Alcohol, units/week	2.85	(1.10)	3.56	(1.24)	<0.001
Current smokers, %		26.5		17.9	0.14
Physical activity in leisure time, %					
Sedentary activity		13.3		17.9	0.20
Moderate activity		64.3		54.7	
Regular activity		22.4		27.4	

Values are means (standard deviation, SD) and percents.
WC, waist circumference.
[a]Numbers may vary due to missing information.
[b]Student's t-test or χ^2 test.
[c]Measurements at Day 1–5 after onset of menstrual cycle.

menstrual cycle between the four groups of women ($P = 0.016$, age-adjusted): parous women with BMI $\geq$ 25 kg/m^2 had a higher average daily level of salivary 17β-estradiol throughout the entire menstrual cycle compared with both nulliparous women ($P = 0.021$, age-adjusted) and parous women ($P = 0.018$, age-adjusted) with BMI < 25 kg/m^2 (Fig. 1B). Also, nulliparous women with BMI $\geq$ 25 kg/m^2 had a higher average daily level of salivary 17β-estradiol throughout the entire menstrual cycle when compared with nulliparous women with BMI < 25 kg/m^2 ($P = 0.039$, age-adjusted). Nulliparous women with WC $\geq$ 77.75 cm had higher average daily levels of salivary 17β-estradiol throughout the entire menstrual cycle compared with nulliparous women with lower WC ($P = 0.017$, age-adjusted). There was a tendency of higher average daily levels of salivary 17β-estradiol throughout the entire menstrual cycle also among parous women with WC $\geq$ 77.75 compared with nulliparous women with lower WC ($P = 0.068$, age-adjusted; Fig. 1C).

Finally, when we stratified women by parity and OC use, we observed no difference in average levels of salivary 17β-estradiol between the four groups of women defined by median split of the two variables ($P = 0.19$, age-adjusted). However, in subgroup analysis, nulliparous women with $\geq$3 years of OC use had higher average daily levels of salivary 17β-estradiol throughout the entire menstrual cycle compared with nulliparous women with fewer years of OC use ($P = 0.050$, age-adjusted; Fig. 1D).

In an analysis using AUC, women with three to five full-term pregnancies had a tendency of higher 17β-estradiol values for the mid-menstrual cycle and mid-cycle intervals, compared with nulliparous women and women with less than two full-term pregnancies

Table II Salivary ovarian hormone concentrations (age-adjusted geometric means, 95% CI) among parous and nulliparous women. The Norwegian EBBA-I study (n = 204)[a].

Ovarian steroids	Parous (n = 98[a])		Nulliparous (n = 106[a])		P-value[b]
	Mean	95% CI	Mean	95% CI	
17β-estradiol, pmol/l					
Overall 17β-estradiol	14.6	(13.0,16.4)	13.4	(12.0,14.9)	0.31
Mid-follicular 17β-estradiol[c]	11.3	(9.53,13.3)	9.69	(8.27,11.4)	0.23
Late follicular 17β-estradiol[d]	17.3	(15.2,19.7)	17.5	(15.5,19.8)	0.91
Overall Progesterone, pmol/l	94.2	(80.9,109.8)	95.6	(82.1,111.2)	0.91

CI, confidence interval.
[a]Number may vary due to missing information.
[b]Linear regression with log transformed hormones as dependent variable.
[c]Aligned cycle Day −10, −6.
[d]Aligned cycle Day −5,−1.

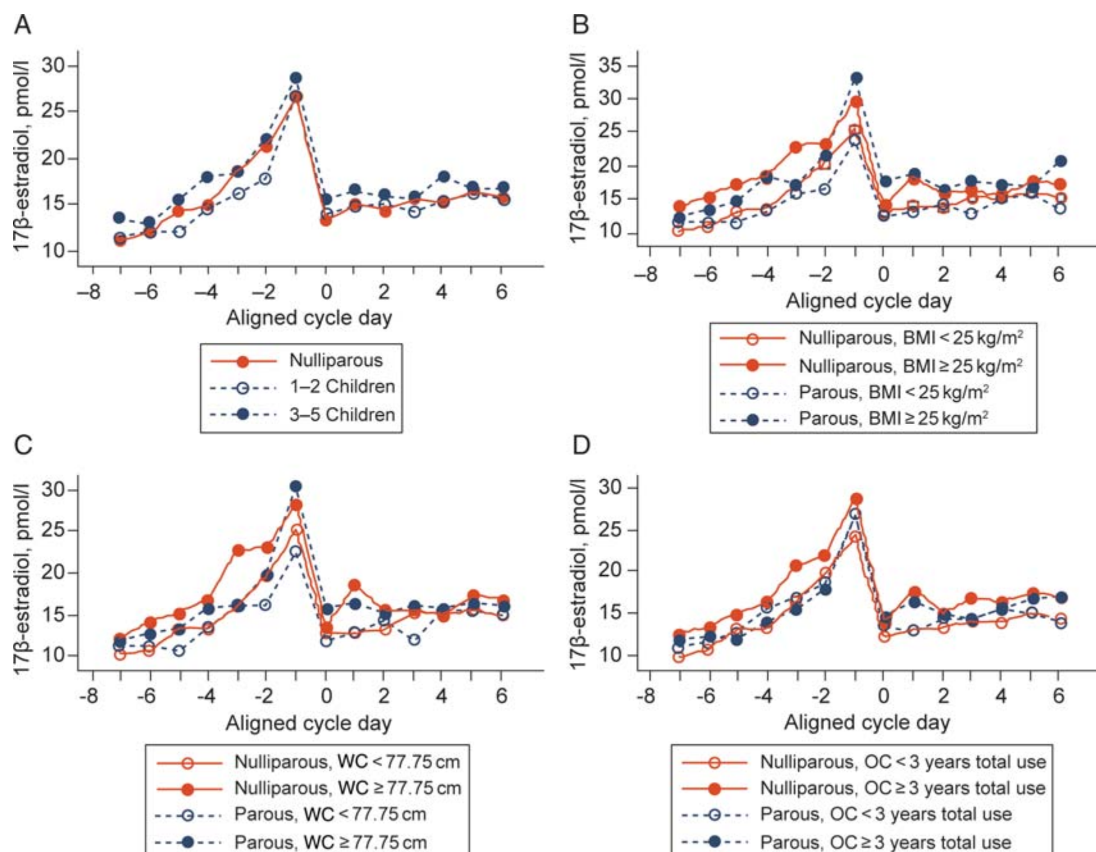

Figure 1 Daily salivary 17β-estradiol concentrations (geometric means) in mid-menstrual cycle for women categorized by (**A**) number of children; nulliparous (n = 99), 1–2 children (n = 74), 3–5 children (n = 17), (**B**) BMI; nulliparous and BMI <25 kg/m^2 (n = 70), nulliparous and BMI ≥25 kg/m^2 (n = 29), parous and BMI <25 kg/m^2 (n = 54), parous and BMI ≥25 kg/m^2 (n = 37), (**C**) WC (median split); nulliparous and waist circumference <77.75 cm (n = 61), nulliparous and waist circumference ≥77.75 cm (n = 38), parous and waist circumference <77.75 cm (n = 36), parous and waist circumference ≥77.75 cm (n = 55), (**D**) OC use (median split); nulliparous and OC <3 years total use (n = 55), nulliparous and OC ≥3 years total use (n = 44), parous and OC <3 years total use (n = 39), parous and OC ≥3 years total use (n = 52).

Table III Estimated cumulative load of salivary ovarian steroids during menstrual cycle according to parity. The Norwegian EBBA-I study (n = 189)[a].

Ovarian steroids	Number of children								P-trend[c]
	0 (n = 99)		1 (n = 36)		2 (n = 37)		3–5 (n = 17)		
	Mean[b]	(95% CI)	Mean[b]	(95% CI)	Mean[b]	(95% CI)	Mean[b]	(95% CI)	
17β-estradiol pmol/l									
Mid-menstrual[d], 14 days	213	(192,236)	220	(205,236)	228	(202,257)	236	(195,286)	0.41
Mid-cycle[e], 6 days	108	(97,120)	112	(104,121)	116	(102,130)	120	(98,145)	0.43
Late follicular[f], 5 days	74	(63,93)	75	(69,81)	76	(67,86)	76	(63,93)	0.79
Progesterone, pmol/l									
Luteal[g], 8 days	969	(866,1083)	934	(860,1013)	900	(789,1026)	867	(703,1070)	0.42

Values are area under curve (AUC, pmol/l).
CI, confidence interval.
[a]Number may vary due to missing information.
[b]Age-adjusted geometric mean.
[c]Linear regression with log transformed hormones as dependent variable.
[d]Aligned cycle Day −7,+6.
[e]Aligned cycle Day −4,+2.
[f]Aligned cycle Day −5,−1.
[g]Aligned cycle Day +2,+9.

(Table III). Nulliparous women also tended to have higher luteal progesterone values compared with all other women in the study, but the association was not statistically significant.

'Menarche-to-first birth' interval and hormonal levels

A shorter interval between menarche and first full-term pregnancy was associated with later age at menarche ($P_{trend} = 0.010$), younger age at first and last full-term pregnancy (both: $P_{trend} < 0.001$), higher parity ($P_{trend} = 0.002$), longer time since last birth ($P_{trend} < 0.001$) and fewer years of education ($P_{trend} = 0.001$) (Table IV).

Overall mean salivary level of 17β-estradiol was inversely related to the length of the time interval between menarche and first birth ($P_{trend} = 0.029$, age-adjusted, Table V). Overall mean salivary progesterone level was not related to the length of the 'menarche-to-first birth' interval ($P_{trend} = 0.34$, age adjusted). The inverse relationship between 'menarche-to-first birth' interval and 17β-estradiol was observed across different weight categories (BMI cut-off for overweight, 25 kg/m^2), age groups (median split, 33 years) and age at menarche (median split, 13.0 years) (data not shown). The age-adjusted geometric mean for maximum peak salivary 17β-estradiol level was 33.0 pmol/l (95% CI, 27.3–39.9) in the lower tertile of 'menarche-to-first birth' interval, 27.2 pmol/l (95% CI, 24.4–30.4) in the mid tertile and 22.5 pmol/l (95% CI, 19.0–26.6) in the upper tertile, equaling a 47% higher maximum peak level of 17β-estradiol for women with the shortest 'menarche-to-first birth' interval when compared with women with the longest interval (data not shown in tables).

When analyzing the 'menarche-to-first birth' interval as a continuous predictor variable in age- and BMI-adjusted linear regression models, we observed a 2.6% ($P = 0.039$) decrease in overall average salivary 17β-estradiol with each 1-year increase in the interval (results not shown). We examined the mean salivary 17β-estradiol concentrations by cycle day and observed a difference among the three 'menarche-to-first birth' interval groups (lower tertile: <10 years; middle tertile: 10–13.5 years; upper tertile: >13.5 years; $P = 0.010$, age-adjusted; Fig. 2).

In analysis of AUC, women with the shortest time interval between menarche and first birth (<10 years) had ~30% higher mid-cycle 17β-estradiol levels ($P_{trend} = 0.050$, age-adjusted) compared with women with the longest intervals (>13.5 years) (Table VI). There was no difference in progesterone values between women in different 'menarche-to-first birth' tertiles ($P_{trend} = 0.99$, age-adjusted).

Discussion

In our study of full cycle profiles of free 17β-estradiol and progesterone among healthy regularly cycling women, we observed no overall association with parity. Interestingly, however, larger waist and longer-term use of OCs were associated with higher daily levels of 17β-estradiol throughout the entire menstrual cycle among nulliparous women. Furthermore, a strong inverse association between the time interval from menarche to first full-term birth and daily salivary 17β-estradiol levels over an entire menstrual cycle among young healthy women with regular menstrual cycles was observed. Women with the shortest 'menarche-to-first birth' interval had 47% higher maximum peak level and 30% higher mid-cycle 17β-estradiol levels compared with the women with the longest interval.

Several previous studies have documented that positive energy balance (Furberg *et al.*, 2005), low physical activity (Verkasalo *et al.*, 2001; Jasienska *et al.*, 2006c; Tworoger *et al.*, 2007) and higher energy resources (Ziomkiewicz *et al.*, 2008) have a positive effect on levels of reproductive ovarian steroids, which in turn improve chances for conception (Lipson and Ellison, 1996; Venners *et al.*, 2006). There are however very limited data on the association

Table IV Characteristics of the study population according to length of the 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-study (n = 98)[a].

Characteristic	Interval between menarche and age at first birth, years						P- trend[b]
	<10 years (n = 30)		10–13.5 years (n = 35)		>13.5 years (n = 33)		
	Mean	(SD)	Mean	(SD)	Mean	(SD)	
Age, years	32.0	(3.10)	32.0	(2.54)	33.0	(1.90)	0.12
Education, total years	13.3	(2.93)	15.6	(2.33)	16.0	(2.30)	0.001
Partnership, total years	9.54	(6.78)	7.35	(5.10)	7.80	(4.51)	0.26
Body composition[c]							
Height, cm	165.9	(5.26)	169.5	(6.96)	165.8	(6.37)	0.76
BMI, kg/m^2	24.8	(3.31)	25.1	(4.10)	25.2	(4.04)	0.77
WC, cm	81.4	(8.11)	83.0	(10.90)	81.2	(11.15)	0.94
Waist to hip ratio	0.80	(0.06)	0.79	(0.06)	0.77	(0.07)	0.07
Reproductive history							
Age at Menarche, years	13.4	(1.22)	13.3	(1.27)	12.5	(1.61)	0.010
Cycle length, days	27.5	(3.00)	28.0	(3.38)	27.8	(2.48)	0.62
Number of children	2.30	(0.95)	1.80	(0.90)	1.61	(0.66)	0.002
Age at first birth, years	20.1	(2.05)	24.8	(1.71)	28.2	(2.26)	<0.001
Age at last birth, years	25.6	(3.29)	27.4	(2.96)	29.7	(2.35)	<0.001
Time since last birth, years	6.41	(3.37)	4.61	(3.12)	3.31	(1.76)	<0.001
Total breastfeeding, months	21.3	(12.76)	17.8	(14.73)	18.9	(9.27)	0.46
Previous use of oral contraceptives, years	4.00	(3.71)	4.71	(3.77)	4.56	(4.13)	0.58
Time since last use of oral contraceptives, years	7.58	(5.42)	7.10	(4.13)	6.71	(3.86)	0.47
Energy intake, kJ/day	7866	(1760)	8145	(2131)	7988	(2084)	0.82
Alcohol, units/week	2.67	(1.01)	3.08	(1.28)	2.77	(0.91)	0.76
Current smokers, %		43.3		20.0		18.2	0.043
Physical activity in leisure time, %							
Sedentary		20.0		8.60		12.1	0.95
Moderate		53.3		74.3		63.6	
Regular		27.7		17.1		24.3	

Values are means (standard deviation, SD) and percents.
WC, waist circumference.
[a]Number may vary due to missing information.
[b]Linear regression or logistic regression.
[c]Measurements at Day 1–5 after onset of menstrual cycle.

between parity and ovarian hormone levels. The present observation supporting no overall associations between levels of 17β-estradiol and progesterone with parity is in agreement with those of former studies (Verkasalo *et al.*, 2001) and importantly, this may lead to further questions related to interacting predisposition and the need of more detailed studies.

Interestingly, nulliparous women with larger waist circumference had higher salivary 17β-estradiol levels compared with nulliparous women with a more narrow waist circumference, supporting that nulliparous women may be more susceptible to lifestyle factors influencing energy balance, abdominal overweight, and metabolic and hormonal profiles. Correspondingly, a positive linear relationship between body fat and estradiol levels throughout an entire menstrual cycle was observed in a parallel study among premenopausal Polish women (Ziomkiewicz *et al.*, 2008). Furthermore, regulation of ovarian hormone levels by nutritional status has been suggested, for example, in studies of women with anorexia nervosa (Miller *et al.*, 2004), and in studies of women in rural communities with seasonal variation in workload (Panter-Brick *et al.*, 1993). Accumulation of excessive abdominal fat is related to insulin resistance with hyperinsulinemia. Insulin stimulates ovarian steroidogenesis and inhibits the hepatic synthesis of SHBG, leading to increased levels of free estradiol (Verkasalo *et al.*, 2001; IARC, 2002; Finstad *et al.*, 2009b). This may explain the positive relation between waist circumference and free 17β-estradiol levels seen in our study, contrary to other studies that have reported inverse associations between waist circumference and total estradiol and its main binding protein, SHBG. However, adjustment for serum SHBG measured at the first visit did not change our

Table V Salivary ovarian hormone concentrations (age-adjusted geometric means, 95% CI) among women in groups of 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-I study (n = 98)[a].

Ovarian steroids	Interval between menarche and age at first birth, years						*P*-trend[b]
	<10 years (n = 30)		10–13.5 years (n = 35)		>13.5 years (n = 33)		
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	
17β-estradiol, pmol/l							
Overall 17β-estradiol	16.2	(13.8,19.3)	14.1	(12.7,15.6)	12.2	(10.4,14.3)	0.029
Mid-follicular 17β-estradiol[c]	11.0	(8.68,14.0)	10.3	(8.98,11.8)	9.63	(7.67,12.1)	0.47
Late follicular 17β-estradiol[d]	18.7	(15.2,23.0)	16.8	(15.0,18.9)	15.1	(12.7,18.1)	0.17
Overall Progesterone, pmol/l	99.2	(77.9,126.3)	90.6	(78.7,104.3)	82.8	(65.9,103.9)	0.34

CI, confidence interval.
[a]Number may vary due to missing information.
[b]Linear regression with log transformed hormones as dependent variable.
[c]Aligned cycle Day −10, −6.
[d]Aligned cycle Day −5, −1.

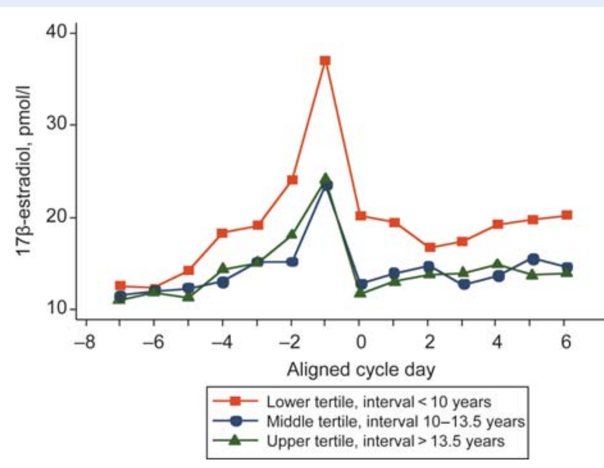

Figure 2 Daily salivary 17β-estradiol concentrations (geometric means) in mid-menstrual cycle for women categorized by tertiles of interval length from menarche to age at first full term birth. Lower tertile; interval <10 years (n = 26). Middle tertile; interval 10–13.5 years (n = 32). Upper tertile; interval >13.5 years (n = 33).

estimates (analysis not presented). In addition, obesity may lead to anovulatory cycles and increased conversion of androgens to estrone in adipose tissue, while the EBBA women were mostly non-obese with normal levels of serum lipids (Furberg *et al.*, 2005). Therefore, our findings support that long-term positive energy balance reflected by larger waist circumference may increase level of free 17β-estradiol in women with regular ovulations, particularly in nulliparous women.

In our study, longer-term use of OCs was associated with higher levels of 17β-estradiol throughout the entire menstrual cycle among nulliparous women. Current use of OCs influences endogenous sex hormone levels, while the association between former use of OCs and circulating levels of 17β-estradiol in adult women is poorly documented. Our findings suggest that the ovarian function in nulliparous women may be more susceptible to long-term suppression by exogenous hormones and possible boosting of 17β-estradiol production after cessation of the pill—we hypothesize that OC exposure may change the physiological set point for the regulation of endogenous hormone levels especially among women that have not experienced a full-term pregnancy. Prospective studies are needed to determine the stability of the hypothalamic–pituitary–ovarian axis phenotype. It might also be that OC use itself is a behavior partially determined by endogenous hormone levels or hormone-related factors.

The present observations that parous women with shorter 'menarche-to-first birth' intervals had a higher parity, lower age at first birth, and higher salivary 17β-estradiol levels than women with longer intervals are indirectly supported by others. Associations between early age at menarche and higher estradiol levels (Bernstein *et al.*, 1991; Emaus *et al.*, 2008a), and higher frequencies of ovulation (Apter *et al.*, 1989) have been observed. Higher energy resources associated with higher estrogens within normal range improve chances for conception (Lipson and Ellison, 1996; Venners *et al.*, 2006). Higher follicular levels of estradiol have been observed in healthy women's menstrual cycles resulting in conception, compared with cycles without conception (Lipson and Ellison, 1996; Venners *et al.*, 2006). Furthermore, the pattern of human sexual behavior is complex but may be explained partly by estradiol levels (Pawlowski and Jasienska, 2005; Durante and Li, 2009). An elevated level of estradiol may lead to more frequent sexual activity, thereby increasing the likelihood of fertilization and parity (Durante and Li, 2009). In the EBBA women, early age at first birth is the main determinant of a shorter menarche-to-first-birth interval rather than late age at menarche—this could be the consequence of conscious choice or a consequence of higher fecundity, or both. On the basis of our observations, we hypothesize that childbearing pattern (i.e. delayed childbirths) in this female population is partly determined by variation in fecundity which again is partly determined by genetic variation in proteins regulating ovarian function as well as gene–environment (i.e. socio-cultural) interactions. Further studies are needed to explore this hypothesis.

Several studies have documented an increase in risk of breast cancer with elevated serum estradiol levels in post-menopausal

Table VI Estimated cumulative load of salivary ovarian steroids during menstrual cycle according length of the 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-I study ($n = 90$)[a].

Ovarian steroids	Interval between menarche and age at first birth						P-trend[c]
	<10 years ($n = 29$)		10–13.5 years ($n = 31$)		>13.5 years ($n = 30$)		
	Mean[b]	(95% CI)	Mean[b]	(95% CI)	Mean[b]	(95% CI)	
17β-estradiol, pmol/l							
Mid-menstrual[d], 14 days	241	(205,286)	218	(197,242)	197	(167,232)	0.11
Mid-cycle[e], 6 days	125	(106,148)	110	(99,122)	96	(82,114)	0.050
Late follicular[f], 5 days	80	(67,96)	72	(65,80)	65	(55,77)	0.12
Progesterone, pmol/l							
Luteal[g], 8 days	857	(700,1049)	858	(757,971)	859	(703,1048)	0.99

Values are area under curve (AUC, pmol/l).
CI, confidence interval.
[a]Number may vary due to missing information.
[b]Age adjusted geometric mean.
[c]Linear regression with log transformed hormones as dependent variable.
[d]Aligned cycle Day −7,+6.
[e]Aligned cycle Day −4,+2.
[f]Aligned cycle Day −5,−1.
[g]Aligned cycle Day +2,+9.

women (Endogenous Hormones and Breast Cancer Collaborative Group, 2002; Prentice *et al.*, 2008). There is evidence for a similar relationship also in premenopausal women (Eliassen *et al.*, 2006) even though the fluctuating level of hormones during the menstrual cycle complicates testing of the hypothesis in premenopausal women (Kaaks *et al.*, 2005).

Our observations suggest that nulliparity in combination with previous OC use and positive energy balance increases the 17β-estradiol levels associated with increased breast cancer risk. Greater BMI and obesity are associated with decreased breast cancer risk in studies of premenopausal women (Friedenreich, 2001; Berstad *et al.*, 2010) while both current and former OC users have an increased risk of breast cancer in recent studies (Lund *et al.*, 2007; Hunter *et al.*, 2010). Low parity is a well-established risk factor for breast cancer (MacMahon *et al.*, 1970; Braaten *et al.*, 2004; World Cancer Research Fund/American Institute for Cancer Research, 2007). A few studies have suggested that giving birth before age 20 is associated with lower risk of breast cancer (MacMahon *et al.*, 1970), whereas first birth at ages above 35 increases breast cancer risk (Trichopoulos *et al.*, 1983), possibly due to increased likelihood of malignant transformations in breast cancer cells in older women. In general, massive differentiation of epithelial breast cells in response to the increased release of ovarian hormones during pregnancy has been proposed as an explanation for both the elevated short-term breast cancer risk after pregnancy, and the extended protective effect of pregnancy (Lambe *et al.*, 1994; Albrektsen *et al.*, 2010).

Furthermore, the longer period of time during which immature breast epithelium cells are exposed to estradiol may cause an elevated risk (Russo *et al.*, 1982; Pike *et al.*, 1993) pointing to the interval between age at menarche and age at first birth, relevant for breast cancer risk (Li *et al.*, 2008; McDougall *et al.*, 2010), and estradiol-receptor-positive tumors in particular (Li *et al.*, 2008). A strong inverse association between the time interval 'menarche-to-first birth' and levels of these hormones suggests that there may be biological mechanisms other than the ovarian steroid pathway that underlie the associations between parity, age at first birth and breast cancer risk. A recent study suggested that a single full term pregnancy decreased the levels of circulating growth hormones (GH), shown to play a role in breast carcinogenesis with insulin-like growth factor I (IGF-I), thus pointing at another potential biological mechanism for protection from breast cancer (Dearth *et al.*, 2010). Further studies are required to fully understand the mechanisms underlying the association between reproductive history and risk of breast cancer.

Our study has the benefit of having collected samples every day over an entire menstrual cycle, rather than only on selected days within a cycle. This allows for estimation of daily free, unbound and biologically active 17β-estradiol and progesterone, and for high quality estimates of full cycle ovarian hormone profiles (Jasisenska and Jasienski, 2008). Furthermore, well-developed and validated methods and assays were used to characterize the women's exposure to free biologically active ovarian steroids and compare levels by aligned cycle days (Lipson and Ellison, 1996). In addition, salivary levels of 17β-estradiol were shown to be quite stable within participants over time (Ellison and Lipson, 1999). Salivary estradiol represents about 1% of the total circulating estradiol, and therefore the absolute values are small in comparison to the serum estradiol levels used in clinical practice. The salivary 17β-estradiol profiles for the EBBA women closely resemble the well-known physiological pattern for cyclic variation in estradiol (Speroff and Fritz, 2005), and large relative differences between individuals were observed (i.e. 47% higher maximum peak level of 17β-estradiol for women with the shortest 'menarche-to-first birth' interval as compared with women with the longest interval). Furthermore, a study evaluating estradiol levels in daily saliva samples for two consecutive menstrual cycles among 12 women observed higher variance in mean estradiol levels between women than between cycles from the same woman, suggesting that a

single menstrual cycle will give reliable estimates in the analysis of interindividual differences (Gann et al., 2001).

The main independent variables in our study (i.e. age at menarche, age at first full-term birth and parity) represent reproductive life milestones, and the women were 25–35 years at participation—these aspects facilitate high reliability of the self-reported data. Furthermore, to minimize false memory of exposures, each woman was interviewed by a trained nurse actively using a detailed lifetime calendar and a list of examples of milestones. In a previous study of the EBBA women, we have shown that age at menarche is associated with 17β-estradiol levels (Emaus et al., 2008a). Also, self-reported birthweight showed to be nearly identical with the birthweight registered at the Medical Birth Registry of Norway (Emaus, 2009).

Our results indicate that lifestyle factors including OC use, excess weight and timing of first birth are associated with ovarian steroid levels among regularly cycling women. Interestingly, long-term positive energy balance may increase 17β-estradiol levels in women with regular ovulations, particularly in nulliparous women. Furthermore, fecundity seems to play a significant role in timing of first childbirth. These findings demonstrate the complexity of the relationships among reproductive factors, levels of ovarian hormones, fecundity and breast cancer risk.

Authors' roles

A.-S.F. participated in collecting the data, suggested the hypotheses, supervised the statistical analyses and manuscript writing. A.M. played a role in data analysis, interpretation and manuscript writing. G.J. was involved in designing the study (in collaboration with Dr I Thune and Dr P. Ellison) and manuscript writing. P.E. designed the study in collaboration with Dr I. Thune and Dr G. Jasienska. T.W. Statistical advisor: supervised the statistical analyses and manuscript writing. V.F., S.E.F. and S.L. commented on the hypotheses and manuscript writing. A.E. participated in collecting the data and manuscript writing. I.T. (the principal investigator of the EBBA-I) designed the study, supervised the statistical analyses and drafted the manuscript. A.I. developed the hypotheses (in collaboration), cleaned the data, performed the statistical analyses and drafted the manuscript.

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Paper III

Associations of daily 17β-estradiol and progesterone with mammographic density in premenopausal women. The Norwegian EBBA-I Study

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Abstract

Purpose: To investigate the associations between daily salivary 17β-estradiol and progesterone concentrations and percent mammographic density among premenopausal women enrolled in the Norwegian Energy Balance and Breast cancer Aspects (EBBA)-I Study and followed over the course of an entire menstrual cycle.

Methods: Among 202 healthy women, aged 25-35 years, daily salivary 17β-estradiol and progesterone concentrations were measured. Computer-assisted breast density readings (MADENA) were obtained from digitized mammograms taken between day 7 and 12 of the menstrual cycle. Multivariable linear and logistic regression models examined the associations between ovarian hormones and percent mammographic density.

Results: Compared with women having a low percent mammographic density (< 28.5%), women with a high percent mammographic density (≥ 28.5%) had 25% higher daily 17β-estradiol concentrations (P = 0.007), and 31% higher daily progesterone concentrations (P = 0.010) across the entire menstrual cycle. Compared with women in the first quartile of overall average daily progesterone concentrations, the odds of high percent mammographic density (≥ 28.5%) increased among women in higher progesterone quartiles (Q4 vs. Q1: Odds Ratio 3.70, 95% Confidence interval 1.35-10.11, P_{trend} = 0.011). These associations were even stronger among nulliparous women with an interaction between parity and average daily progesterone in the luteal phase (P = 0.017). We also observed strong associations between serum concentrations of ovarian hormones and percent mammographic density.

Conclusion: Daily 17β-estradiol and progesterone were strongly associated with percent mammographic density in premenopausal women, and could in part explain the association of breast density with increased breast cancer risk.

Introduction

Mammographic density reflects the relative amount of connective and epithelial tissue and fat in the breast, and is a strong and independent risk factor for breast cancer (1-2). Women with the highest percent mammographic density (> 75%) have four to six times greater breast cancer risk compared to women with less dense breasts (3-5). Mammographic density declines at menopause and has been positively associated with both combined estrogen plus progestin use (6-7), and endogenous estrogen levels in postmenopausal women (8). In contrast, knowledge about the association between endogenous ovarian hormones and mammographic density among premenopausal women is limited and inconsistent (9-11).

A woman's lifetime exposure to estrogens has a large cumulative effect on her risk of getting breast cancer (12), and estrogen plays a key role in breast carcinogenesis (13-14). Recently, an association between hormone exposure and mammographic density was observed in women who later developed breast cancer (15). Thus, studies including daily levels of endogenous estrogen and progesterone may be valuable in clinical practice (8). However, other factors may influence mammographic density alone or in combination with ovarian hormones. Parity has been observed to be inversely related to mammographic density (16-19), possibly due to lobular differentiation of breast tissue during pregnancy (19-20).

Previously, in the Norwegian Energy Balance and Breast cancer Aspects (EBBA-I) Study, when analyzing the association between metabolic risk profile and ovarian hormones, we observed a crude positive association between salivary progesterone concentrations and mammographic density using a modified Wolfe's classification (21). Thus, the main aim of the present study was to examine the associations between daily 17β-estradiol and progesterone concentrations across the menstrual cycle among premenopausal women and percent mammographic density assessed by the more accurate computer-assisted method (MADENA) (22).

Materials and methods

Participants and study design

The women participating in the Norwegian EBBA-I Study (2000-2002) were recruited through local media campaigns. A total of 204 women, 25-35 years were included and met the following eligibility criteria; regular menstrual cycles (cycle length: 22-38 days within the previous three

months), no use of any medication, no pregnancy or lactation or use of steroid contraceptives over the previous 6 months, and no gynecological or chronic medical conditions (e.g. diabetes) (21-23). Information including parity and lifestyle factors was collected using questionnaires and interviews. All questionnaires were checked for inconsistencies by one trained nurse. The participants underwent clinical examination at the Clinical Research Center, University Hospital of North Norway (UNN), Tromsø, at three scheduled visits during their menstrual cycle: first visit between day 1 and 5 after onset of the menstrual cycle (early follicular phase), second visit between day 7 and 12 (late follicular phase), and third visit between day 21 and 25 (late luteal phase). Two women were excluded due to missing mammographic data, leaving data from 202 premenopausal women available for the present study.

Daily 17β-estradiol and progesterone

Women collected daily morning saliva samples at home for one entire menstrual cycle, and sampling started on the first day of bleeding (21, 24-25). Hormone assays were run in the Reproductive Ecology Laboratory, Harvard University, Cambridge, USA (25). Saliva samples from 20 consecutive days (reverse cycle days -5 to -24; with -1 implicating the last day of the menstrual cycle) of the women's menstrual cycle was used for 17β-estradiol analysis, and saliva samples from14 consecutive days (reverse cycle days -1 to -14) was used for progesterone analysis (21).

Alignment of the cycles for analysis was based on the identification of the mid-cycle estradiol drop (aligned cycle day 0), which provides a good estimate of the day of ovulation (25). Identification of the drop in salivary 17β-estradiol concentration was not satisfactory for 14 women, and their cycles were not aligned. Overall average salivary 17β-estradiol and progesterone were calculated for all women, and additional indices of average hormone concentrations were calculated for 188 women: "luteal" index (aligned cycle days 0 to +6) and "mid-menstrual" index (aligned cycle days -7 to +6). Maximum peak level corresponds to the highest measured hormone concentration during the aligned cycle (day -1).

Serum samples and clinical examination

Fasting morning blood samples were obtained at all three visits. Fresh serum 17β-estradiol and progesterone concentrations were measured by direct immunometric assay (Immuno-1, Bayer Diagnostics, CITY, Country), sex hormone-binding globulin (SHBG) was measured by an

immunometric method (both Diagnostic Products Corporation (DPC)-Bierman GmbH, Bad Nauheim, Germany), and total cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase, and high-density lipoprotein cholesterol (HDL-C) was quantified by direct assay using enzymes modified by polyethylene glycol and dextran sulfate, at the Department of Clinical Chemistry, UNN.

Anthropometric measurements were performed with participants wearing light clothing and no footwear. Height was measured to the nearest 0.5 cm, and weight to the nearest 0.1 kg on an electronic scale. Body mass index (BMI) was calculated in kg/m^2. At the second visit participants underwent a full-body scan to estimate total percent body fat, using dual energy X-ray absorptiometry (DEXA, DPLX-L 2288, Lunar Radiation Corporation, Madison, Wisconsin, USA). Blood pressure was measured (21, 23).

Mammographic density

Bilateral two-view mammograms were obtained at the second visit (day 7-12) using a standard protocol (Furberg 2005). Left craniocaudal mammograms were digitized and imported into a computerized mammographic density assessment program (MADENA) (22). The total breast area was defined on the mammographic image using a special outlining tool. The region of interest (ROI) was then outlined. The mammogram reader used a tinting tool to apply yellow tint to pixels considered to represent areas of mammographic density. The MADENA software estimated the total number of pixels and the number of tinted pixels in the ROI. "Absolute mammographic density" represents the number of the tinted pixels within the ROI, and "percent mammographic density" is the ratio of absolute mammographic density to the total breast area (area of ROI) multiplied by 100. The mammograms were read in four batches, with an equal number of mammograms in each batch. A duplicate reading of 26 randomly selected mammograms from two of the batches showed a Pearson's correlation coefficient of 0.97. The reader was blinded to all characteristics of the study population. The density assessments were conducted by one of the authors (G.U.), and the breast areas were outlined by a research assistant trained by G.U.

Ethical considerations

All the participants signed an informed consent form and the study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Statistical analysis

Percent mammographic density was dichotomized into high and low density breasts, using the median percent mammographic density (28.5%) as the cut point. Student's t-test, Pearson's chi-squared test or linear regression was used to compare means and proportions of selected characteristics by median split of percent mammographic density (< 28.5%, and ≥ 28.5%). The adjusted odds ratios (ORs) of having above median percent mammographic density (≥ 28.5%) were estimated using logistic regression models. We also stratified the models by parity (parous, nulliparous). Both salivary 17β-estradiol and progesterone concentrations were included as categorical variables (quartiles) in separate analyses. Tests of interaction between parity and hormone concentrations were conducted by including cross product terms in the models. Previous oral contraceptive use was not an independent predictor of percent mammographic density when adjusting for age and BMI, and was not included in the final models. Generalized estimating equation models were used to assess the associations of daily salivary 17β-estradiol and progesterone concentrations with median split of percent mammographic density. Age, BMI, parity and indicator variables of aligned cycle day were included when appropriate as covariates in models. Hormone data were log transformed prior to statistical analyses, but untransformed hormone values are presented in participants' characteristics to facilitate readability. All statistical tests were two-sided using a 5% significance level. Statistical analyses were conducted with STATA version SE 11.0 (Stata Corporation, College Station, Texas, USA).

Results

The participating premenopausal women had a mean age of 30.7 years, mean age at menarche of 13.1 years and none were current users of steroid hormones. The mean (standard deviation) and median (range) percent mammographic density were 29.8% (19.0 %) and 28.5% (15.3 - 78.9%), respectively (data not shown). Nulliparous women had a higher mean percent mammographic density than parous women, 35% versus 25%, respectively ($P < 0.001$, adjusted for age and BMI) (data not shown).

Table 1. Characteristics of the premenopausal women by median split of percent mammographic density. The Norwegian EBBA-I study (n=202)[a]

	Percent mammographic density				
	< 28.5 % (n=101)		≥ 28.5 % (n=101)		*P* value[b]
Age, years	31.5	(31.0,32.1)	29.9	(29.3,30.5)	*<0.001*
Reproductive factor					
Age at menarche, years	12.9	(12.6,13.1)	13.4	(13.1,13.6)	*<0.011*
Cycle length, days	27.9	(27.3,28.5)	28.7	(28.0,29.3)	*0.069*
Number of children	1.35	(1.11,1.59)	0.49	(0.32,0.65)	*<0.001*
Clinical measurements[c]					
Height, cm	167.3	(166.2,168.4)	166.7	(165.3,168.1)	*0.48*
BMI, kg/m^2	26.1	(25.4,26.9)	22.7	(22.1,23.2)	*<0.001*
Total body fat (DEXA), %	37.9	(36.6,39.3)	30.5	(29.2,31.8)	*<0.001*
Systolic blood pressure, mmHg	115.5	(113.0,117.9)	111.0	(109.2,112.9)	*0.004*
Diastolic blood pressure, mmHg	72.1	(70.5,73.7)	69.8	(68.2,71.3)	*0.039*
Daily saliva samples, fasting pmol/l					
Estradiol, overall	17.6	(15.8,19.3)	18.5	(16.8,20.2)	*0.44*
Estradiol, luteal[e]	16.8	(15.0,18.7)	18.3	(16.4,20.2)	*0.28*
Progesterone, overall	119.8	(107.5,132.2)	141.6	(127.3,155.9)	*0.023*
Progesterone, luteal[e]	109.3	(96.5,122.0)	132.4	(117.4,147.3)	*0.020*
Serum samples, fasting					
Total cholesterol[c], mmol/l	4.55	(4.39,4.70)	4.36	(4.20,4.51)	*0.087*
HDL-C[c], mmol/l	1.47	(1.40,1.54)	1.60	(1.54,1.66)	*0.005*
Estradiol, late luteal[d], pmol/l	374.9	(342.6,407.1)	488.0	(445.3,530.7)	*<0.001*
Progesterone, late luteal[d], nmol/l	31.0	(27.6,34.4)	41.0	(36.7,45.2)	*<0.001*
SHBG, late luteal[d], nmol/l	48.9	(44.7,53.2)	56.9	(52.7,61.0)	*0.009*
Oral contraceptive use, years	4.47	(3.74,5.20)	3.22	(2.51,3.94)	*0.017*
Alcohol consumption, units/week	3.11	(2.89,3.33)	3.33	(3.05,3.61)	*0.22*
Energy intake, kJ/day	7943	(7559,8327)	8273	(7907,8638)	*0.22*
Sedentary leisure activity, %		17.8		13.9	*0.44*
Current smokers, %		26.7		17.8	*0.13*

Notes: To obtain conventional unit divide by the following conversion factor: 3.671, estradiol (pg/ml); 3.18, progesterone (ng/ml). Data are expressed as unadjusted mean (95% CI) or percentage.
[b]Student's *t* or chi square test.
[c]Serum samples in early follicular phase: day 1-5 after onset of menstrual cycle, except total tissue fat (DEXA) measured at day 7-12 (late follicular phase) after onset of the menstrual cycle.
[d]Serum samples in late luteal phase: day 21-25 after onset of menstrual cycle.
[e]Daily saliva samples in luteal phase: aligned cycle day 0, +6 (n = 188).
Abbreviations: CI, confidence interval; BMI, body mass index; DEXA, dual-energy x-ray absorptiometry; HDL-C, high-density lipoprotein cholesterol; SHBG, sex hormone-binding globulin

In the present study, younger age ($P < 0.001$), older age at menarche ($P = 0.011$), and lower parity ($P < 0.001$) were positively associated with percent mammographic density (Table 1). Furthermore, BMI and body fat (% DEXA) were inversely related to percent mammographic density (all $P < 0.001$). Unadjusted serum concentrations of estradiol ($P < 0.001$), progesterone ($P < 0.001$), and SHBG ($P < 0.009$) in the luteal phase were positively associated with percent mammographic

density (Table 1). The association with serum SHBG disappeared after adjustment for age and BMI (P = 0.52) (data not shown). Higher unadjusted salivary concentrations of overall average and luteal phase progesterone were positively associated with percent mammographic density (P = 0.023 and P = 0.020, respectively) (Table 1).

17β-estradiol concentration and percent mammographic density

When comparing premenopausal women with high (≥ 28.5%) versus low (< 28.5%) percent mammographic density, we observed 25% higher daily salivary 17β-estradiol concentrations in the mid-menstrual phase (*P* = 0.007), 23% higher daily salivary 17β-estradiol concentrations in the luteal phase (*P* = 0.024), and a 21% higher maximum peak salivary 17β-estradiol concentration (*P* = 0.031), all adjusted for age and BMI (Table 2 and Figure 1A). Moreover, women in the higher quartiles of overall average salivary 17β-estradiol concentrations had higher adjusted odds of high percent mammographic density (≥ 28.5%) compared to women in the lower quartile (Q4 vs.Q1: OR 2.69, 95% CI 0.97-7.51, P_{trend} = 0.031) (Table 3 and Figure 1A). A similar relationship was observed in the luteal phase (Q4 vs. Q1: OR 2.58, 95% CI 0.91-7.33, P_{trend} = 0.058) (Table 3 and Figure 1A). Serum estradiol concentrations in the late follicular phase and the late luteal phase were positively associated with percent mammographic density (*P* = 0.050 and *P* = 0.006, respectively) (Table 2).

When stratifying by parity, high (≥ 28.5%) versus low (< 28.5%) percent mammographic density was associated with 35% higher daily salivary 17β-estradiol concentrations in the mid-menstrual phase among nulliparous women (*P* = 0.011) (Figure 1C). Nulliparous women in the higher quartiles of salivary 17β-estradiol concentrations in the luteal phase, had higher adjusted odds of high percent mammographic density (≥ 28.5%) compared to nulliparous women in the lower quartile (P_{trend} = 0.010) (data not shown). A similar relationship was observed for overall average salivary17β-estradiol concentrations among nulliparous women (P_{trend} = 0.063) (data not shown). No association between salivary 17β-estradiol concentration and percent mammographic density was observed among parous women (Figure 1C). No interaction between salivary 17β-estradiol concentration and parity was observed.

Table 2. Age and BMI adjusted means of 17β-estradiol and progesterone concentrations in saliva and serum for the premenopausal women by median split of mammographic density*. The EBBA-I Study (n = 202)

	Percent mammographic density		
	< 28.5 % (n=101)	≥ 28.5 % (n=101)	*P* value[a]
Estradiol, pmol/l			
Daily saliva samples			
Overall	12.70 (11.41,14.13)	15.65 (14.06,17.41)	*0.012*
Mid-menstrual[b]	13.29 (11.94,14.80)	16.91 (15.13,18.90)	*0.007*
Luteal[c]	12.65 (11.26,14.21)	15.52 (13.76,17.50)	*0.024*
Maximum peak[d]	24.43 (21.83,27.35)	29.63 (26.38,33.27)	*0.031*
Serum samples			
Early follicular[e]	147.6 (135.4,159.9)	143.6 (131.4,155.9)	*0.68*
Late follicular[f]	371.4 (315.6,429.5)	461.1 (401.3,523.4)	*0.050*
Late luteal[g]	378.9 (342.3,416.5)	459.9 (422.1,499.8)	*0.006*
Progesterone			
Daily saliva samples, pmol/l			
Overall	84.59 (75.15,95.21)	102.89 (91.41,115,80)	*0.033*
Luteal[c]	81.02 (71.03,92.41)	111.66 (97.45,127.94)	*0.010*
Serum samples, nmol/l			
Luteal[g]	32.2 (28.1,36.3)	39.7 (35.6,43.9)	*0.020*

Notes: To obtain conventional unit divide by the following conversion factor: 3.671, estradiol (pg/ml); 3.18, progesterone (ng/ml).
*Reported as arithmetic mean for serum progesterone and geometric means for all other samples.
[a]Linear regression and generalized estimating equation.
[b]Daily saliva samples in mid-menstrual phase: aligned cycle day -7,+6 (n = 188).
[c]Daily saliva samples in luteal phase: aligned cycle day 0,+6 (n = 188).
[d]Maximum peak:aligned cycle day -1 (n = 188).
[e]Serum samples in early follicular phase: day 1-5 after onset of menstrual cycle.
[f]Serum samples in late follicular phase: day 7-12 after onset of menstrual cycle.
[g]Serum samples in late luteal phase: day 21-25 after onset of menstrual cycle.
Abbreviations: CI, confidence interval; BMI, body mass index.

Progesterone concentration and percent mammographic density

When comparing premenopausal women with high (≥ 28.5%) versus low (< 28.5%) percent mammographic density, we observed 31% higher daily salivary progesterone concentrations in the luteal phase of the menstrual cycle, adjusted for age and BMI (P = 0.010) (Table 2 and Figure 1B). Women in the higher quartiles of overall average salivary progesterone concentrations had higher adjusted odds of high percent mammographic density (≥ 28.5%) compared to women in the lower quartile (Q4 vs. Q1: OR 3.70, 95% CI 1.35-10.11, P_{trend} = 0.011) (Table 3 and Figure 1B). A similar association was observed in the luteal phase with highest adjusted odds for mammographic density

≥ 28.5% observed in the third progesterone quartile (Q3 vs. Q1: OR 4.92, 95 % CI 1.67-14.44, P_{trend} = 0.032), (Table 3 and Figure 1B). Serum progesterone concentration in the luteal phase was positively associated with mammographic density (≥ 28.5%) (P = 0.020), (Table 2).

When stratifying by parity, high (≥ 28.5%) versus low (< 28.5%) percent mammographic density was associated with 36% higher daily salivary progesterone concentrations across the entire luteal phase among nulliparous women (P = 0.03) (Figure 1D). Furthermore, nulliparous women in the higher quartiles of overall average salivary progesterone concentrations, had higher adjusted odds of high percent mammographic density (≥ 28.5%) compared to nulliparous women in the lower quartile (P_{trend} = 0.028) (data not shown). A similar association was observed in the luteal phase among nulliparous women (P_{trend} = 0.005) with highest adjusted odds for mammographic density ≥ 28.5% observed in the third progesterone quartile (data not shown). There was an interaction between parity and luteal phase concentrations of salivary progesterone (P = 0.017).

Table 3. Odds Ratios (95% CI) for high percent mammographic density (≥ 28.5 %) by quartiles of daily salivary 17β-estradiol and progesterone concentrations among the premenopausal women. The EBBA-I Study (n = 202)

	Q1	Q2	Q3	Q4	P_{trend}[a]
17β-estradiol, pmol/l					
Overall	1.0 (ref)	1.38 (0.54-3.55)	2.37 (0.87-6.44)	2.69 (0.97-7.51)	*0.031*
Luteal[b]	1.0 (ref)	1.43 (0.53-3.89)	2.00 (0.72-5.51)	2.58 (0.91-7.33)	*0.058*
Progesterone, pmol/l					
Overall	1.0 (ref)	2.35 (0.88-6.28)	2.99 (1.07-8.34)	3.70 (1.35-10.11)	*0.011*
Luteal[b]	1.0 (ref)	3.63 (1.24-10.61)	4.92 (1.67-14.44)	3.04 (1.06-8.74)	*0.032*

[a]Logistic regression, adjusted for age, body mass index, and parity (parous, nulliparous)
[b]Daily saliva samples in luteal phase: aligned cycle day 0, +6, (n = 188).
Abbreviations: CI, confidence interval.

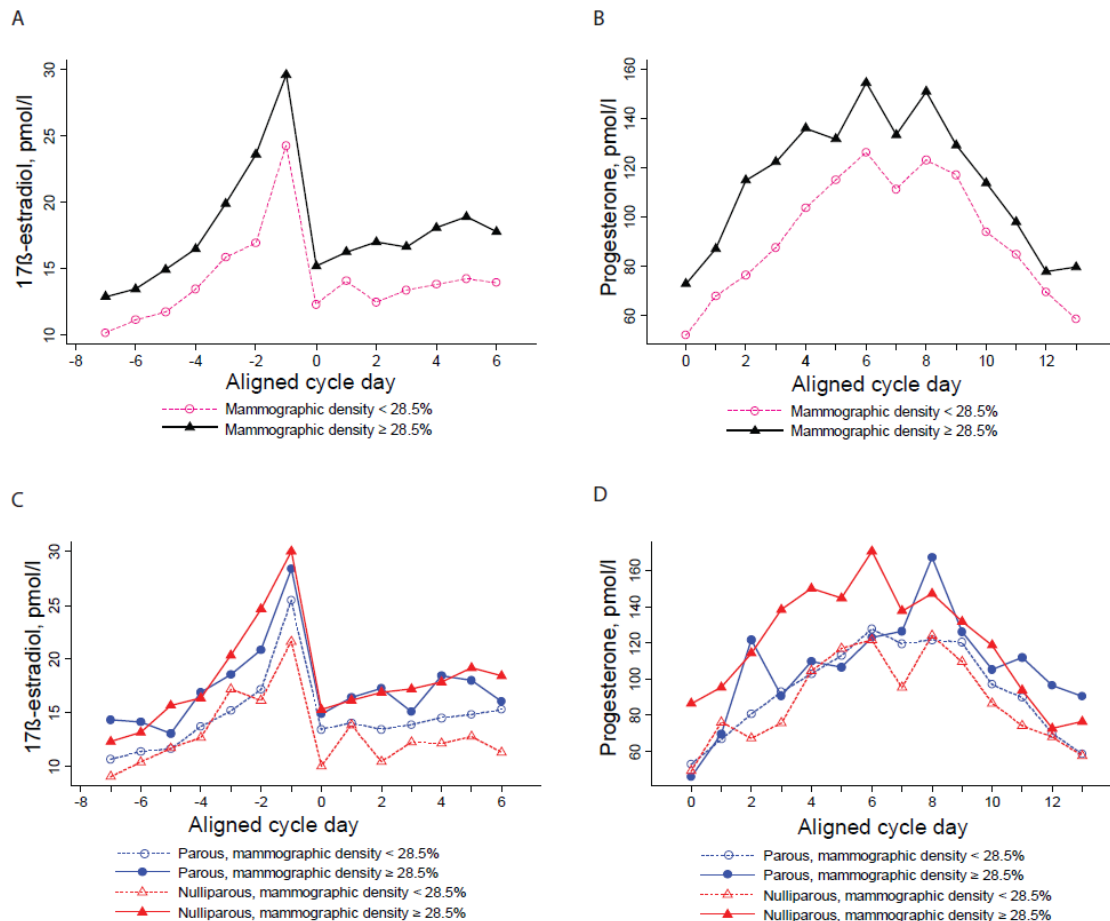

Figure 1. Age and body mass index adjusted daily salivary concentration (geometric means) of A) C) 17β-estradiol (aligned cycle day -7, +6), and B) D) progesterone (aligned cycle day 0, +13) for A) B) premenopausal women categorized by median split of percent mammographic density; < 28.5 % (n=96), ≥ 28.5 % (n=92) and C) D) premenopausal women categorized according to parity and percent mammographic density (median split); nulliparous and percent mammographic density < 28.5 % (n=31), nulliparous and percent mammographic density ≥ 28.5 % (n=66), parous and percent mammographic density < 28.5 % (n=65), parous and percent mammographic density ≥ 28.5 % (n=26). The EBBA-I Study.

Discussion

Our finding of a strong positive association between daily endogenous estrogen and progesterone concentrations and percent mammographic density in premenopausal women extends previous studies. Most of the previous studies have focused on the relationship between sex steroids and mammographic density among postmenopausal women, while studies examining the associations between endogenous ovarian hormone profiles over the entire menstrual cycle and mammographic density among premenopausal women have been sparse. However, our observation that women with a high percent mammographic density (≥ 28.5%) had 25% higher daily 17β-estradiol

concentrations, and 31% higher daily progesterone concentrations, across the entire menstrual cycle compared with women having a low percent mammographic density (< 28.5%) is partly supported. Interestingly, a positive association between follicular phase serum estradiol concentrations and percent mammographic density among women was observed (mean age: 42.4 years) (11), and serum concentration of progesterone was positively associated with percent mammographic density among premenopausal women (10). Furthermore, total urinary estrogen metabolites were positively associated with percent mammographic density in premenopausal women (26), and a direct association was observed between preovulatory and luteal phase urinary estrone glucuronide and percent mammographic density (27). In contrast to our results, the magnitude of the association was reduced after adjustment for BMI, but these women were mostly parous with mean age 48.4 years (27). In addition, urinary estrone glucuronide may reflect different biological pathways and mechanisms than serum and salivary estradiol.

The suggested effect of both endogenous estrogen and progesterone on mammographic density in premenopausal women in our study is supported by reports from randomized trials including postmenopausal women, showing that combined estrogen plus progesterone use is associated with larger changes in percent mammographic density than estrogen use alone (6-7, 14). These data also hypothesize that progesterone may be an even stronger predictor of mammographic density than estrogen (6-7, 14, 28). So far, reports from observational studies among postmenopausal women are conflicting (8), however, in some studies, mammographic density increased with higher endogenous estrogen (29-30) and progesterone concentrations (29).

Furthermore, our results are consistent with the hypothesis that a positive association between circulating free estrogen and progesterone and breast cancer risk may be mediated, in part, by increasing mammographic density. Recently, changes in mammographic density by hormone exposure were observed to be stronger in women who later developed breast cancer (15). Whether mammographic density or a specific threshold of mammographic density in early adulthood is predictive of breast cancer risk later in life is not known. Breast tumors have been shown to arise predominantly within the radiodense areas of the breast (31). Thus, mammographic density and levels of endogenous estradiol and progesterone at a given age may in combination be important markers for breast cancer risk later in life. Our findings support the hypothesis that lowering levels

of estradiol and progesterone (32-35) in young women may reduce mammographic density and improve diagnostics and breast cancer risk assessment (36-37).

We observed that the associations between ovarian hormones and breast density were stronger among nulliparous women than among parous women, suggesting that the relationship between ovarian hormones and breast density may vary by parity among premenopausal women. Such an interaction may be explained by the fact that percent mammographic density decreases after first full-term pregnancy (19). Parity-induced molecular changes in growth factors, cell fate, p53 activation or induction of a specific genomic signature in the breast may be involved (38-39). Thus, we hypothesize that relative to the breast tissue of parous women, the breast tissue of nulliparous women may be more susceptible to higher endogenous ovarian hormone concentrations influencing percent mammographic density. Also, others have suggested that any effect of estrogen and progesterone on breast tissue may vary due to a woman's reproductive status (40) of importance for breast carcinogenesis (14, 41-42). Interestingly, single nucleotide polymorphisms on genes involved in the estrogen pathway have been associated with mammographic density among premenopausal nulliparous women only (43).

Our results are strengthened by the collection of daily salivary measurements of unbound estradiol and progesterone concentrations across an entire menstrual cycle (25, 44-45), following strict procedures and validated methods (21, 24-25). Mammographic density was assessed within a narrow time frame in late follicular phase (between days 7-12) (46). The computer-assisted method used to quantify mammographic density has been shown to give a superior prediction of breast cancer risk compared with qualitative methods (2, 4). All mammograms were read by one experienced blinded reader, and the assessed mammographic density was negatively associated with age, BMI and parity (47-48). However, the small sample size in the present study and few previous reports underline the need for further studies.

In summary, the present findings support the hypothesis that both daily endogenous estradiol and progesterone concentrations are strongly associated with percent mammographic density in premenopausal women in a dose-response manner. Furthermore, our results suggest that nulliparous premenopausal women may be more susceptible to ovarian hormones in prediction of

mammographic density. Interventions to lower estradiol and progesterone in premenopausal women, such as aerobic physical activity and low-fat diets, could aid in reducing density thereby reducing breast cancer risk and improving sensitivity of premenopausal mammogram screening. However, more studies including ovarian hormones across the menstrual cycle are needed to confirm and improve the clinical implications of these findings.

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Appendix A

- General questionnaire
- Lifetime calendar
- Questionnaire: previous use of hormonal contraceptives
- Photographic booklet of food items, pre-coded food diary and questionnaire: diet
- Instructions and daily logbook of physical activity and saliva sampling

THE EBBA SURVEY

English translation; Mrs. Anne Clancy and Mrs. Anniken Telnes Iversen

We know little about the direct causes of various types of cancer. For that reason it is uncertain what each one of us can do to reduce our risk of getting cancer. The main purpose of this survey is to improve our knowledge of these illnesses in order to prevent them. We would like you to answer questions about your lifestyle and health. You will be making an important contribution by providing us with good knowledge that can be put to practical use in helping to prevent these serious diseases.

The survey has been approved by the Regional Board of Research Ethics. The answers you give will be treated in strict confidence and will only be used for research purposes. The information may later be compared with information from other public health registers in accordance with the rules laid down by the Data Inspectorate and the Regional Board of Research Ethics.

Thank you in advance for helping us.

Yours sincerely,
Inger Thune , M.D.

CONFIDENTIAL

GENERAL INFORMATION

Municipality of birth ___________________________________
(If you were born outside Norway, give name of country instead of municipality.)

Marital status (tick the appropriate box)
- Single ☐
- Married/living together ☐
- Widow ☐
- Separated/divorced ☐
- Other ☐

How many years schooling/training have you had in total?
(Include everything from primary school upwards - middle/ secondary school, vocational training/higher education/university)

__________ years

How many years of your active working life have you mainly
- done housework (including maternity leave)? __________ years
- been employed full time outside the home? __________ years
- been employed part time outside the home? __________ years

Do you have brothers and/or sisters? ☐ Yes ☐ No

If yes, how many? Sisters? __________ Brothers?__________

How many children had your mother given birth to before you were born? __________

Which ethnic group do your ancestors belong to?
(Parents/grandparents) (Tick the most appropriate boxes)
- Norwegian ☐
- Sami ☐
- Other European ☐
- Finnish ☐
- Asian ☐
- Other; give details ____________________ ☐

HEIGHT/WEIGHT

You might not know your height and weight from childhood onwards. We would nevertheless like you to try to answer.

Birth:	Weight	____	grams	Height	____	cm
At age 18:	Weight	____	kg	Height	____	cm
Today:	Weight	____	kg	Height	____	cm

How would you describe your body compared to children your own age when you were growing up? (Tick one box for each age group)

	Much thinner	Thinner	Normal	Fatter	Much fatter
Pre-school	☐	☐	☐	☐	☐
Grades 1-6	☐	☐	☐	☐	☐
Grades 7-9 (13-16 years)	☐	☐	☐	☐	☐

MENSTRUATION/PREGNANCIES/BREAST-FEEDING

How old were you when you had your first menstrual period?
______ years ______months

How long did it take before your periods became regular?
(Tick the most appropriate box)
- One year or less ☐
- More than 1 year ☐
- Never ☐
- Cannot remember ☐

How have your periods been? (Tick one box)
- Always regular ☐
- Usually regular ☐
- Irregular ☐

What was/is the usual number of days between periods?

(From day 1 of one period to day 1 of the next period)

__________ days

Have you had children? ☐ Yes ☐ No

If yes, have you ever been treated for nausea/ vomiting during pregnancy? ☐ Yes ☐ No

If you have had children, fill in year of birth and number of months you breast-fed each child (this should be completed also for children who died at birth or later in life).

Child no.	Year of birth	Number of months of breast-feeding

PHYSICAL ACTIVITY

HOUSEWORK

THE LAST 12 MONTHS

Imagine an average week of housework during the last 12 months (all types of work in the home, including caring for and dressing children/others in need of care). We have divided housework into 4 levels of activity. For each activity level, you should fill in the **number of days per week** and average **time per day** (in minutes) spent on such work.

Level of activity for housework is defined as	Days per week	Minutes per day
1 = Mostly sedentary work (sewing, writing)		
2 = Light work, carried out standing up, sitting down, or while walking slowly (e.g. cooking, dusting)		
3 = Fairly heavy work: You perspire a little and your heart beats a little faster; (e.g. doing laundry, vacuum cleaning, caring for/dressing others)		
4 = Heavy physical work: You perspire profusely and your heart beats quickly; (e.g. heavy cleaning)		

THROUGHOUT YOUR LIFE

Housework changes with age and circumstances. Using the same activity levels as above (1-4), we would like you to fill in for each age given: the average number of **days per week and hours per day** you carried out activities at each level.

Activity level:		Age				
		10-14 years	15-19 years	20-24 years	25-29 years	30-34 years
1=Sedentary work	Days per week					
	Hours per day					
2=Light work	Days per week					
	Hours per day					
3=Fairly heavy work	Days per week					
	Hours per day					
4=Heavy physical work	Days per week					
	Hours per day					

LEISURE ACTIVITIES (EXERCISE, HIKING, SPORTS)

THE LAST 12 MONTHS

What kind of physical activity have you done in your leisure time in the last 12 months? If your activity level varies a lot, for instance between summer and winter, then give an average. (Tick only the most appropriate box.)

1= Reading, watching TV or doing other sedentary activities? ☐

2= Walking, riding a bicycle or other forms of exercise at least four hours a week? (Including walking or riding a bicycle to and from work, Sunday walks, etc.) ☐

3= Exercising, doing heavy gardening, etc.? (Note that the activity must take up **a minimum of four hours** a week.) ☐

4= Exercising intensively or doing competitive sports regularly several times a week? ☐

LEISURE ACTIVITIES AND CODES

Below is a list of various leisure activities. We would like you to note the activities you have participated in. In the form below please indicate how much time (number of sessions per month and time per session) you spent doing each activity. Using the four activity levels given for leisure activities below, tick the level that best fits each activity.

1. Reading books/watching TV
2. Walking to/from work/school, taking walks, walking with a pram
3. Hiking in the forest/mountains, hunting
4. Jogging/running
5. Riding a bicycle to/from work/school (including exercise bike)
6. Swimming (and diving/deep-sea diving)
7. Handball/basketball/ (ball-game similar to baseball or rounders)/football (soccer)
8. Volleyball
9. Tennis/badminton/squash
10. Golf/bowling/curling
11. Athletics: javelin, discus, shot-put/high and long jump
12. Gymnastics/aerobics/exercising/ dancing/ballet
13. Health studio/weight lifting
14. Skipping or similar
15. Skiing: cross-country – recreational/competitive
16. Downhill skiing/ Telemark ski-ing/snowboarding
17. Tobogganing/kick-sledging/skating (ice and roller skates)
18. Horse riding
19. Rowing/paddling/sailing
20. Picking berries/ mushrooms/fishing
21. Other

ACTIVITY LEVELS FOR LEISURE ACTIVITIES:

1 = Mainly sedentary.

2 = Light training: You do not sweat and your heart does not beat faster.

3 = Moderate training: You sweat a little and your heart beats a little faster.

4 = Hard/heavy training: You sweat profusely and your heart beats fast.

Activity Type of activity	Months per year	Average no. of sessions per month	Average time per session (minutes)	Activity level			
				1	2	3	4

THROUGHOUT YOUR LIFE

Look again at the list of various leisure activities. Please mark the activities you have participated in. Then give the age at which you participated in the activity, and indicate how often and for how long you participated (months per year, time per week and per session). Tick the **one** activity level that best fits each activity, using the four levels given for leisure activities above.

Activity and code	Age at start	Age at end	Months per year	Hours per week	Average time per session (minutes)	Activity level			
						1	2	3	4

WATCHING TV, SITTING, REST IN YOUR FREE TIME.

How many minutes or hours of your free time per 24 hours do you usually devote to the following activities? Calculate an average for the last 12 months.

	Number of hours	Number of minutes
Resting, sleeping	_________	_________
Listening to music/radio	_________	_________
Watching TV/videos	_________	_________
Meals, coffee/tea	_________	_________
Reading/writing	_________	_________
Conversation (incl. phone calls)	________	_________
Handicrafts, hobbies	_________	_________

WORK/SCHOOL ACTIVITIES

THE LAST 12 MONTHS

Have you been in paid employment/a student during the last 12 months? ☐ Yes ☐ No

If yes:

Months working/studying in the last 12 months __________ months

Workdays per week __________ days

Working hours per day __________ hours

What level of physical activity do you normally have at work/school now? (Tick the box that you feel fits best)

1 = Mostly sedentary work
(e.g. office work) ☐

2 = Work that requires a lot of walking
You do not perspire and your heart does not beat faster (e.g. shop assistant, teacher, hairdresser) ☐

3 = Work that requires a lot of walking and lifting
You perspire a little and your heart might beat faster (e.g. nurse/assistant nurse, postman/woman) ☐

4 = Heavy manual labour.
You perspire quite a bit and your heart beats fast (e.g. heavy lifting, farming, heavy-duty care) ☐

Imagine an average week of work/school activity in the last 12 months. Here too we have divided the activities into the same 4 activity levels as above. For each activity level indicate the number of months per year, hours per week and average number of hours per day, which you dedicated to the activity.

Type of work Activity level	Months per year	Hours per week	Average per day
1=seated			
2=Standing and walking			
3=Walking and carrying			
4=Heavy			

TRAVEL TO/FROM WORK/SCHOOL

This question relates to travel between home and work in the last 12 months.

How do you usually get to/from work? Give an average for a month for the numbers of times you use:

Car _______
Bus/tram/train/boat _______
Bicycle _______
On foot _______

How long does it usually take you to get to/from work?
Add up the time you spend getting to work and back for each mode of transportation. If relevant, give the time it takes you to walk to/from car park/bus stop, etc.

Car _______ minutes
Bus/tram/train/boat _______ minutes
Bicycle _______ minutes
On foot _______ minutes

THROUGHOUT YOUR LIFE

We would first like you to indicate the schools you have attended/jobs you have had. If you have worked at home in a capacity other than as a housewife, e.g. farming, childminding, sewing, these should be given. You might have had several jobs in the same time period, e.g. while working part-time. Please fill in how old you were when you started and finished each job, and indicate how much time you usually spent/spend at various activity levels: number of months per year, days per week, and hours per day. Tick the activity level most appropriate for each job, using the 4 activity levels above. Remember exercise hours/breaks at school.

Jobs/ schools	Age at start	Age at end	Months per year	Days per week	Hours per day	Activity level			
						1	2	3	4

MEDICINES

Please tick **yes** for the medicines you have used occasionally (however little) and **no** for those you have never used. If you tick **yes**, try to remember what age you were the first time you used the medicine and the number of times per month you use it now.

	Yes	No	Age first time	No of times per month
Hypertensive drugs	☐	☐	_______	_______
Painkillers	☐	☐	_______	_______
Acetylsalicylic acid/Albyl E				
Antidepressants	☐	☐	_______	_______
If yes, which ones				
Others	☐	☐	_______	_______
If yes, which ones				

Please tick YES for those of the following medicines you use regularly (daily, almost daily)

	Yes
Sleeping pills	☐
Painkillers	☐
Hypertensive drugs	☐
Antidepressants	☐
Other medicines	☐
If yes, which ones _________________	
Homoeopathic/herbal medicines	☐
If yes, which ones _________________	

CANCER IN THE FAMILY

Have any of your close biological relatives had cancer? ☐ Yes ☐ No

If yes, which type of cancer has occurred in your maternal and paternal family?

Maternal family	*Type of cancer*	No	Don't know
Mother	____________	☐	☐
Mother's mother	____________	☐	☐
Mother's father	____________	☐	☐
Aunt	____________	☐	☐
Uncle	____________	☐	☐
Others, indicate relationship _________	____________	☐	☐
Paternal family			
Father	____________	☐	☐
Father's father	____________	☐	☐
Father's mother	____________	☐	☐
Uncle	____________	☐	☐
Aunt	____________	☐	☐
Others, indicate relationship __________	____________	☐	☐

LIFESTYLE

Have you ever smoked on a daily basis? ☐ Yes ☐ No

If yes, how many cigarettes did you smoke each day on average? (Tick one box for each age group.)

Number of cigarettes per day

	0	1-4	5-9	10-14	15-19	20-25	25 +
12-14 years	☐	☐	☐	☐	☐	☐	☐
15-19 years	☐	☐	☐	☐	☐	☐	☐
20-24 years	☐	☐	☐	☐	☐	☐	☐
25-34 years	☐	☐	☐	☐	☐	☐	☐

Do you smoke every day now? ☐ Yes ☐ No

If yes, how many cigarettes a day?

How many habitual smokers did you live with at the following ages? (Tick one box in each line.)

Number of persons :	None	1	2	3 or more	Don't know
Childhood	☐	☐	☐	☐	☐
15-19 years	☐	☐	☐	☐	☐
20-24 years	☐	☐	☐	☐	☐
25-34 years	☐	☐	☐	☐	☐

Do you currently live with someone who smokes? ☐ Yes ☐ No

If yes, how many cigarettes do they normally smoke per day when you are with them? __ cigarettes

Have you ever worked in smoke-filled workplaces? ☐ Yes ☐ No

If yes, for how long altogether? _______ years

Have you ever drunk alcohol? ☐ Yes ☐ No

If yes, how many glasses of wine, ½ litres of beer, or measures of spirits did you drink on average per month at the following ages? (Tick one box in each line.)

	Never/ rarely	1 pr. month	2-3 pr. month	1 pr. week	2-4 pr. week	5-6 pr. week	1+ pr. day
15-19 years	☐	☐	☐	☐	☐	☐	☐
20-24 years	☐	☐	☐	☐	☐	☐	☐
25-34 years	☐	☐	☐	☐	☐	☐	☐

Are you currently a teetotaller? ☐ Yes ☐ No

If no, how many glasses of wine, ½ litres of beer, or measures of spirits have you drunk on average per month or per week in the last 12 months? (Tick one box in each line.)

	Never rarely	1 pr. month	2-3 pr. month	1 pr. week	2-4 pr. week	5-6 pr. week	1+ pr. day
Beer (1/2 litre)	☐	☐	☐	☐	☐	☐	☐
Wine (glasses)	☐	☐	☐	☐	☐	☐	☐
Fortified wine (0,4 dl)	☐	☐	☐	☐	☐	☐	☐
Spirits (measures)	☐	☐	☐	☐	☐	☐	☐

Your comments:

____

____

____

May we have your permission to contact you again at a later stage to update this information?

☐ Yes ☐ No

Thank you for taking part in the survey!

Personal calendar of events in life

It can be difficult to remember what one has done previously, what one was occupied with during different periods of life and how physically active one has been. It may help to have a calendar in front of you and maybe even fill in events, before you attempt to answer the questionnaire.

Year	What happened?	Suggested events you can fill in.
1964		
1965		- Date of birth
1966		
1967		
1968		
1969		
1970		
1971		-Started primary school
1972		
1973		
1974		
1975		
1976		-Started secondary school
1977		
1978		-First menstrual period
1979		
1980		
1981		- Confirmation
1982		
1983		-Started other schools
1984		
1985		
1986		-Work
1987		
1988		-Gave birth, number of children
1989		
1990		-Other events
1991		Arrival of siblings
1992		Travels
1993		Wedding
1994		Family events (Mother's /father's 50th
1995		birthday etc.)
1996		
1997		
1998		
1999		
2000		
2001		
2002		

THE EBBA SURVEY

English translation; Mrs. Anne Clancy

CONTRACEPTIVE PILLS/INJECTABLE CONTRACEPTION/HORMONE-RELEASING INTRAUTERINE DEVICE **Serial number__________**

	Yes	No
Have you ever used the pill, mini pill included?	☐	☐
Have you ever used injectable contraception?	☐	☐
Have you used a hormone-releasing intrauterine device ("coil")?	☐	☐
If you have given birth, did you use the pill, an injectable contraceptive or intrauterine device before you gave birth the first time?	☐	☐
Have you been given the pill, an injectable contraceptive or intrauterine device for reasons other than contraception?	☐	☐
Have you, for medical reasons, been recommended to discontinue use of the pill, injectable contraceptive or intrauterine device?	☐	☐

We would like more detailed information about your usage of the pill, injectable contraceptive or intrauterine device.

Can you remember which periods of your life you used the pill, injectable contraceptive or intrauterine device continuously?
How old were you when you started?
How old were you when you stopped?
Over how long a period did you use the same brand of the pill, injectable contraceptive or intrauterine device?
What was the name of the pill, injectable contraceptive or intrauterine device (see enclosed list of brand names and numbers)? If you cannot recall the brand, write "unsure" in the space provided for the brand.

Period	Age started	Age stopped	Continuously		Contraceptive pill	
			Year	Month	Number	Brand
First						
Second						
Third						
Fourth						
Fifth						
Sixth						
Seventh						

Brands of the pill, injectable contraception or intrauterine device?

Monophasic pills

Recommended use: 1 tablet daily for 21-22 days, then a break or placebo tablets for 6-7 days.

(1) **Follimin**

(2) **Microgynon**

(3) **Eugynon**

(4) **Marvelon**

(5) **Yasmin**

(6) **Diane**

(7) **Loette**

Multiphasic pills

Usual use: comes in calendar blister packs.

(8) **Synfase**

(9) **Trinordiol**

(10) **Trionetta**

Progestagen-only pills

(11) **Conludag**

(12) **Exlutona**

(13) **Microluton**

Injectable contraception

(14) **Depo-provera**

Hormone-releasing intrauterine device

(15) **Levonova**

Other

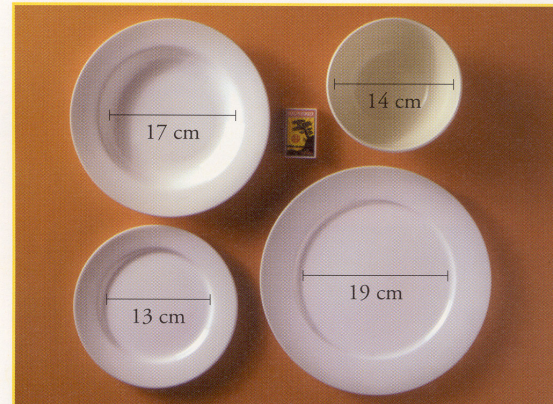
(16) Name the brand

Unsure

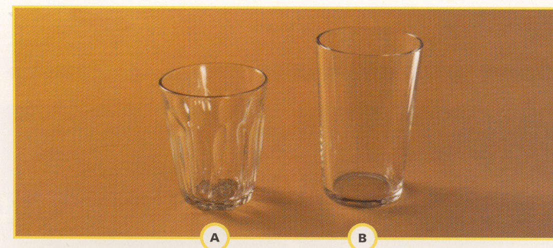
(17)

Bildehefte med porsjonsstørrelser

DETTE BILDET VISER STØRRELSEN PÅ TALLERKENENE SOM ER BRUKT I BILDEHEFTET

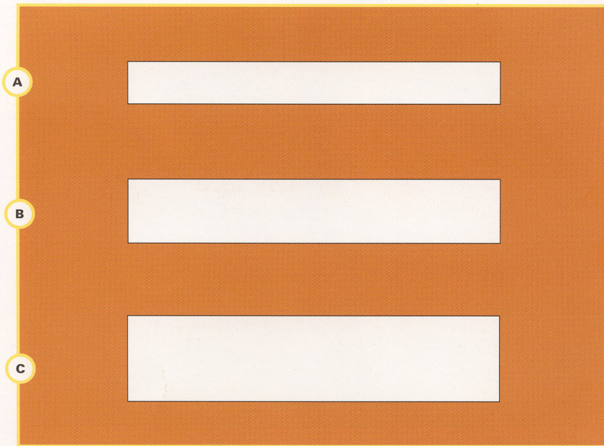


1. GLASS

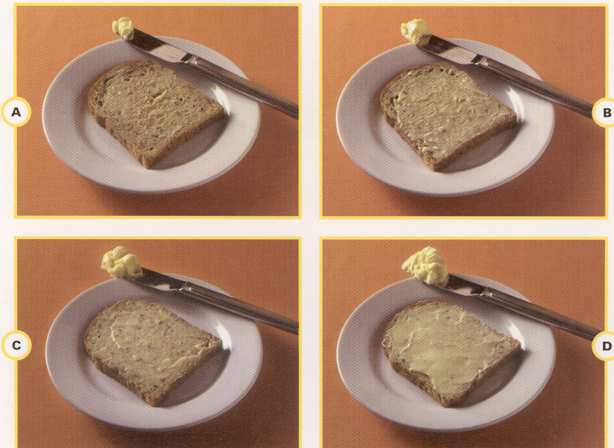


2. KOPPER

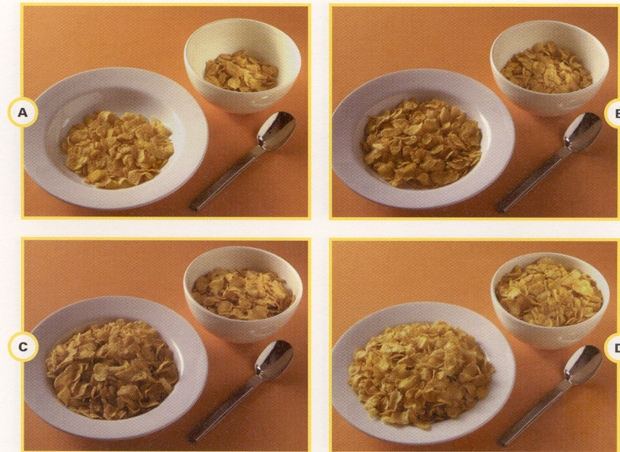
3. BRØD TYKKELSE



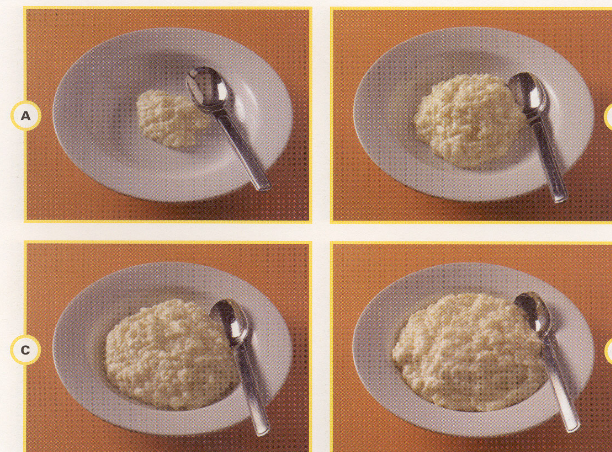
4. SMØR/MARGARIN PÅ BRØDET



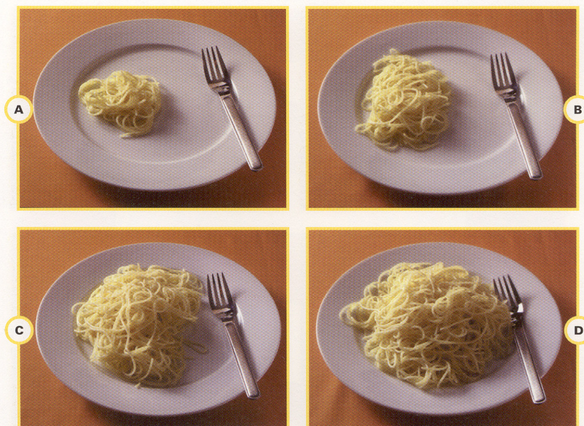
5. CORNFLAKES (FROKOSTBLANDING)



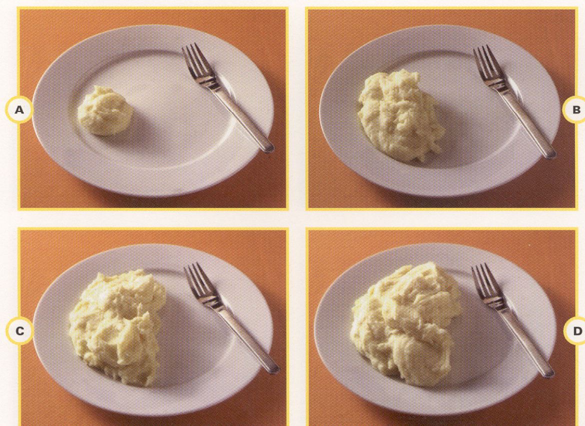
6. GRØT



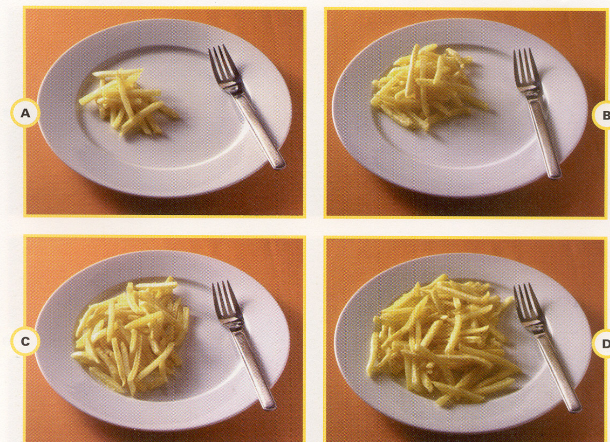
7. SPAGHETTI / PASTA (RIS)



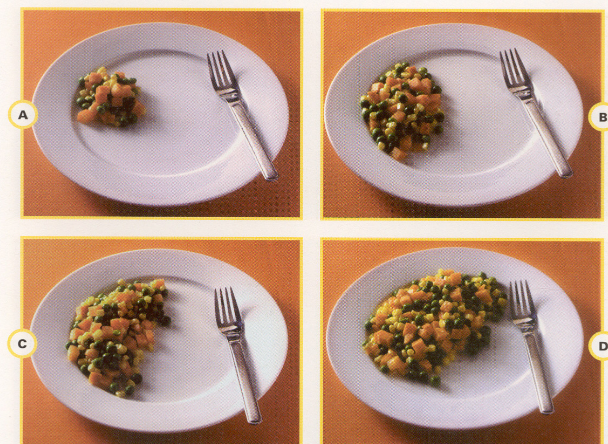
8. POTETMOS



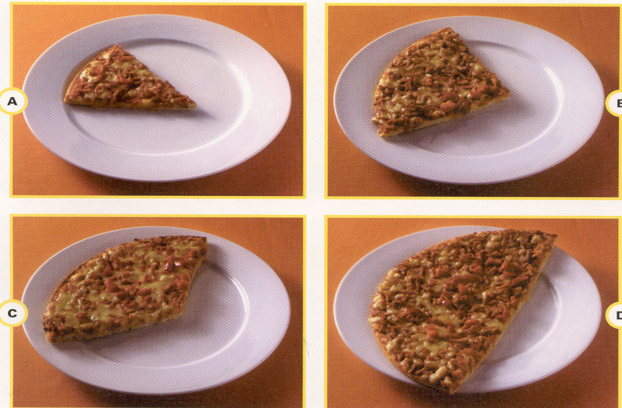
9. POMMES FRITES (STEKT POTET)



10. GRØNNSAKSBLANDING (RÅKOST)

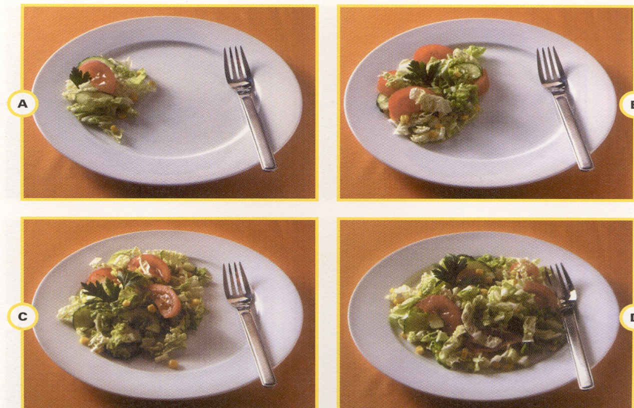


13. PIZZA, TREKANTSTYKKER

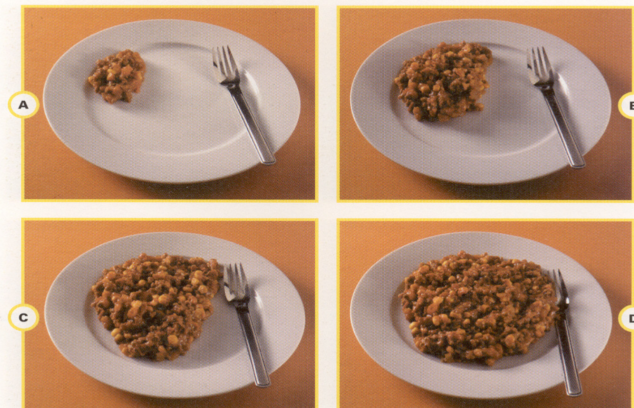


14. PIZZA, FIRKANTSTYKKER

11. SALAT



12. KJØTTSAUS (LAPSKAUS)



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UTVIKLET AV:

STATENS RÅD FOR ERNÆRING OG FYSISK AKTIVITET

SNT Statens næringsmiddeltilsyn

INSTITUTT FOR ERNÆRINGSFORSKNING

Picture Booklet illustrating size of portions

English translation: Anne Clancy

This photo illustrates plate sizes used in the booklet

1.Glasses

Picture A	Picture B
150 g	230 g

2.Cups

Picture A	Picture B	Picture C	Picture D
110 g	160 g	240 g	270 g

3. Thickness of slices of bread

A B C

4. Butter/margarine on bread

Picture A	Picture B	Picture C	Picture D
3 g	6 g	9 g	12 g

5. Cornflakes (Cereals)

Picture A	Picture B	Picture C	Picture D
10 g	30 g	57 g	86 g

6 Porridge

Picture A	Picture B	Picture C	Picture D
50 g	200 g	350 g	500 g

7. Spaghetti/pasta (rice)

Picture A	Picture B	Picture C	Picture D
34 g	68 g	160 g	250 g

8. Mashed potatoes

Picture A	Picture B	Picture C	Picture D
60 g	205 g	355 g	500 g

9. French fries

Picture A	Picture B	Picture C	Picture D
30 g	60 g	90 g	120 g

10. Mixed vegetables (raw grated vegetables)

Picture A	Picture B	Picture C	Picture D
40 g	80 g	120 g	160 g

11. Salad

Picture A	Picture B	Picture C	Picture D
33 g	52 g	100 g	175 g

12. Meat Stew

Picture A	Picture B	Picture C	Picture D
50 g	200 g	350 g	500 g

13. Pizza , triangular slices

Picture A	Picture B	Picture C	Picture D
56 g	114 g	165 g	270 g

14. Pizza, square slices

Picture A	Picture B	Picture C	Picture D
52 g	112 g	165 g	270 g

15. Filet of fish

Picture A	Picture B	Picture C	Picture D
36 g raw	*102 g raw*	*16 0 g raw*	*195 g raw*
27 g fried	84 g fried	134 g fried	166 g fried

16. Dessert (ice cream)

Picture A	Picture B	Picture C	Picture D
38 g	64 g	97 g	139 g

Some foods that we have mentioned, in the questionnaire, but that are not illustrated in the picture booklet.

Cereals **(conversion factor from cornflakes to whole grain muesli cereal is 4,6)**

Picture A	Picture B	Picture C	Picture D

46 g	138 g	262 g	396 g

Rice (conversion factor from spaghetti to rice is 1,3)

Picture A	Picture B	Picture C	Picture D
44 g	88 g	208 g	325 g

Fried potato (conversion factor from french fries to fried potatoes id is 1,33)

Picture A	Picture B	Picture C	Picture D
40 g	80 g	120 g	160 g

Raw grated vegetables (conversion factor from mixed vegetables to raw grated vegetables is 0,7)

Picture A	Picture B	Picture C	Picture D
28 g	56 g	84 g	112 g

Chocolate pudding (conversion factor from ice cream to chocolate pudding is 2)

Picture A	Picture B	Picture C	Picture D
76 g	128 g	194 g	278 g

ID. number:________

THE EBBA SURVEY

(Breast cancer and lifestyle)
English translation; Mrs. Anne Clancy

DIETARY QUESTIONS

Day:_____________ **Date:**_____________ **Reg day:**_____________

Was today a normal day, or an unusual one, considering what you ate and drank?

Normal day ☐ **Unusual day** ☐

The reason for it being an unusual day: _

Where do I find the different foodstuffs in the dietary questions?

Drinks	page 2	Potatoes/rice/pasta	page 7
Yogurt	page 2	Vegetables	page 7
Bread	page 3	Sauce/salad dressings	page 7
Cereals and porridge	page 3	Ice cream/dessert	page 8
Sandwich fillings	page 4	Fruit/berries	page 8
Meat and meat dishes	page 5	Cakes/biscuits	page 9
Fish and fish dishes	page 6	Chocolate/sweets	page 9
Other warm dishes/ salads	page 6	Snacks	page 9

Cod-liver oil/dietary supplements

1 tea spoon = 5 ml

	Number	(Morning Midday afternoon evening) All together today
Cod-liver oil	tea-spoon	
Cod-liver capsules	No.	
Soluble multivitamins	tea-spoon	
(eg. biovit sanasol)	tea-spoon	
Multivitamin tablets (vitaplex, vitamineral)	no.	
Fluoride tablets	no	
Iron pills (9 mg)	no.	
Vitamin C tablets	no.	
Others - describe type and amount:		

Drinks

Use no. 1 and 2 in the photo series to estimate the size of cups and glasses
1/2 liter = 2,5 glasses

	Number	Morning	Midday	Afternoon	Evening
Water/sparkling water	glass				
Full cream milk (sweet/sour)	glass				
Semi-skimmed milk (sweet/sour)	glass				
Extra semi-skimmed milk	glass				
Skimmed milk (sweet/sour)	glass				
Drinking yogurt	glass				
Chocolate milk	glass				
Cocoa	cup				
Juice /nectar	glass				
Soft drink with sugar	glass				
Soft drink without sugar	glass				
Tea	cup				
Ice tea with sugar	glass				
Coffee	cup				
Artificial sweetener	No..				
Sugar for tea/coffee	tea spoon				
Milk for tea/coffee	soup spoon				
Beer	½ litter				
Wine	glass				
Spirits	shorts/ cocktails				
Others – describe type and amount:					

Yogurt

	Number	Morning	Midday	Afternoon	Evening
Natural yogurt plain	cup (175 ml)				
Fruit yogurt	cup (175 ml)				
Low fat yogurt	cup (150 ml)				
Yogurt and muesli	cup (with muesli)				
Others – describe type and amount:					

Bread

Use no.3 in the photo series to estimate bread thickness
1 slice of bread=1/2 bread roll

	Number	Morning	Midday	Afternoon	Evening
White bread/bread roll	of slices photo series 3				
Semi-wholemeal bread	of slices photo series 3				
Wholemeal bread	of slices photo series 3				
Baguette / Ciabatta	pcs.				
Crisp bread	pcs..				
Flat potato cake	pcs.				
Hamburger bread/Hot dog bread roll	pcs.				
Thin wafer crisp bread	pcs.				
Others – describe type and amount:					

What type of butter/margarine do you spread on your bread?

For the amount of butter/margarine on bread, use no. 4 in the photo series (chose A,B,C or D)
1 slice of bread = ½ roll = 2 biscuits

	Number	Morning	Midday	Afternoon	Evening
Butter	of slices:				
Soft margarine	of slices:				
Light margarine	of slices:				
Hard margarine	of slices:				
Others – describe type and amount:	of slices:				

Cereals and porridge

Use no. 5 and 6 in the photo series (chose A,B,C or D)

	Number of portions	Morning	Midday	Afternoon	Evening
Oatmeal porridge					
Oat flakes					
Muesli with added sugar					
Muesli (unsweetened)					
Cornflakes					
Frosties/ choco pops					
Others – describe type and amount:					

Milk/sugar/jam used with cereals and porridge

1 soup spoon = 3 teaspoons (15ml)

	Number	Morning	Midday	Afternoon	Evening
Full cream milk (sweet/sour)	dl				
Semi-skimmed milk (sweet/sour)	dl				
Skimmed milk (sweet/sour)	dl				
Jam, marmalade	teaspoons				
Jam, low sugar	teaspoons				
Sugar	teaspoons				
Others – describe type and amount:					

Sandwich fillings/spreads

Fill in the number of slices of bread. Indicate amount of fillings/spreads according to slices of bread. If you have two fillings on the same slice of bread, mention both. (eg. 1 White cheese, full cream and 1 ham). If you have eaten only the filling and not bread, please note how many slices of bread you could have used the filling on.
1 slice of bread = ½ roll = 1 crisp bread = 2 biscuits

	Number	Morning	Midday	Afternoon	Evening
Cheese					
White cheese, full cream (27% fat)	of slices				
White cheese, reduced fat (16% fat)	of slices				
Brown full fat cheese,	of slices				
Brown cheese, reduced fat	of slices				
Cream cheese (eg. Philadelphia)	of slices				
Cream cheese low fat (eg. Philadelphia light)	of slices				
Desert cheese (eg. Brie, Camembert)	of slices				
Sandwich meats/spreads					
Luncheon roll	of slices				
Ham, cured ham, low fat luncheon roll	of slices				
Salami, smoked sausage, mutton sausage	of slices				
Liver patè	of slices				
Liver patè, low fat	of slices				
Fish fillings/spreads					
Caviar	of slices				
Smoked salmon/trout	of slices				
Mackerel in tomato sauce	of slices				
Sardines, marinated herrings, anchovies	of slices				
Jam/other sweet spreads					
Jam, marmalade	of slices				
Jam, marmalade (low sugar)	of slices				
Honey	of slices				
Peanut butter	of slices				
Chocolate fillings/spreads	of slices				
Other sandwich fillings					
Egg, boiled/fried	of slices				
Salads with mayonnaise	of slices				
Salads with mayonnaise, low fat	of slices				
Tomatoes	of slices				
Bananas	of slices				
Mayonnaise	of slices				
Mayonnaise, low fat	of slices				
Others – describe type and amount:					

Meat and meat dishes

	Amount	Morning	Midday	Afternoon	Evening
Sausages					
Frankfurters	no.				
Frankfurters, low fat	no.				
Sausages, dinner type	no.				
Sausages, dinner type, low fat	no.				
Minced meat dishes / pasta / pizza					
Meat balls (made from minced beef)	pcs.				
Meat balls (made from minced pork)	pcs.				
Taco (with minced meat and salad)	filled taco				
Kebab / Pita bread (with meat and salad)	filled pita				
Minced meat sauce / tomato sauce with minced meat	photo series 12				
Pasta with tomato sauce (without meat)	photo series 7				
Pasta with white sauce	photo series 7				
Lasagna	piece (10 x 5 cm)				
Pizza, square slices	photo serie 14				
Pizza, triangular slices	photo serie 13				
Lean meat					
Beef /lam/ pork	pcs.				
Chops (beef, lam, pork)	pcs.				
Roast (beef, lam, pork)	slices				
Ham	slices				
Grilled chicken	1/4 chicken				
Chicken filet	no. of filets				
Bacon	slices				
Stew/ casserole dishes					
Rice dishes/risotto)	photo series 12				
Mutton and cabbage stew / mutton with white gravy sauce	photo series 12				
Norwegian stew (meat and vegetable stew)	photo series 12				
Other meat and vegetable stews	photo series 12				
Liver dishes	photo series 12				
Others – describe type and amount:					

Fish and fish dishes

	Number	Morning	Midday	Afternoon	Evening
Minced fish					
Fish balls	No.				
Fish cakes/fish loaf	No. / slices				
Fish					
Cod/coalfish/Norway haddock (boiled)	pcs.				
Cod/coalfish/Norway haddock (fried)	photo series 15				
Salmon/trout/halibut (boiled)	pcs.				
Salmon/trout/halibut (fried)	photo series 15				
Herring/mackerel (boiled)	pcs.				
Herring/mackerel (fried)	photo series 15				
Flounder/wolf fish (boiled)	pcs.				
Flounder/wolf fish (fried)	photo series 15				
Fish dishes/fish in batter					
Fish fingers	pcs.				
Fried fish (in batter)	pcs. (10x10 cm)				
Fish casserole/fish soup	dl				
Fish pie	dl				
Shrimps	dl				
Others – describe type and amount:					

Other hot dishes/salads

	Number	Morning	Midday	Afternoon	Evening
Rice porridge	photo series 6				
Pancakes	pcs.				
Meat soup	soup bowls				
Soup (eg. cauliflower soup, tomato soup)	soup bowls				
Egg, boiled, fried, omelette.	number of eggs				
Cheese pie/quiche	pcs.				
Mixed salad with cheese, meat or shrimps	photo series 11				
Salad with pasta and cheese, meat or shell fish	photo series 11				
Vegetarian dish – describe type and amount:					
Others – describe type and amount:					

Potatoes/rice/pasta

	Number	Morning	Midday	Afternoon	Evening
Boiled potatoes	No.				
Baked potatoes	no.				
Mashed potatoes	photo series 8				
French fries	photo series 9				
Fried potatoes	photo series 9				
Potato salad	tea-spoons				
Rice, boiled	photo series 7				
Pasta boiled (eg. spaghetti, macaroni, tagliatelle)	photo series 7				
Others – describe type and amount:					

Vegetables

	Number	Morning	Midday	Afternoon	Evening
Carrots	pcs.				
Turnips	slices				
Broccoli, cauliflower	dl				
Cabbage	dl				
Raw-grated vegetables (mix of several vegetables)	photo series 10				
Vegetable mix	photo series 10				
Mixed salad (eg. chinese leaves, corn, tomato, cucumber)	photo series 11				
Tomato/pepper/fried onion	slices				
Others – describe type and amount:					

Sauce/salad dressings

1 soup spoon = 3 tea spoons

	Number	Morning	Midday	Afternoon	Evening
White sauce	soup spoons				
Gravy	soup spoons				
Melted butter/margarine	soup spoons				
Tomato sauce (without meat)	soup spoons				
Béarnaise sauce	soup spoons				
Salad dressing (eg. Thousand Island)	soup spoons				
Salad dressing low fat (eg. Thousand Island light)	soup spoons				
Sour Cream 35 % fat	soup spoons				
Sour Cream 20 % fat	soup spoons				
Mayonnaise	soup spoons				
Mayonnaise low fat	soup spoons				
French dressing	soup spoons				
Others – describe type and amount:					

Ice cream/desserts

	Number	Morning	Midday	Afternoon	Evening
Ice cream (eg. crushed caramel, vanilla)	photo series 16				
Ice lolly/cone	no.				
Jelly	photo series 16				
Pudding (eg. Crème-Brule, chocolate pudding)	photo series 16				
Creamed rice, fromage, cloudberries in whipped cream	photo series 16				
Cream	soup spoons				
Whipped cream	soup spoons				
Chocolate sauce/caramel sauce	soup spoons				
Custard	dl				
Others – describe type and amount:					

Fruit/berries

	Number	Morning	Midday	Afternoon	Evening
Apple/pear	no.				
Banana	no.				
Orange	no.				
Mandarin oranges	no.				
Grapes	no.				
Peach/nectarine	no.				
Fresh/frozen berries	dl.				
Others – describe type and amount:					

Cakes/biscuits

	Number	Morning	Midday	Afternoon	Evening
Sweet buns	pcs.				
Danish pastries	pcs.				
Waffles	pcs.				
Apple pie/cut-cake	slices				
Chocolate cake	slices				
Cream cake	slices				
Macaroon cake, nut cake	slices				
Plain sweet biscuits (eg. Marietta)	pcs.				
Fancy biscuits (eg. Maryland Cookies)	pcs.				
Oat meal biscuits	pcs.				
Plain biscuits	pcs.				
Water biscuits	pcs.				
Biscuits with salt (Ritz)	pcs.				
Others – describe type and amount:					

Chocolate/Sweets

	Number	Morning	Midday	Afternoon	Evening
Milk chocolate	chocolate bar (100 g)				
Marzipan covered with chocolate	chocolate bar (65 gram)				
Assorted chocolates	pcs.				
Snickers, Mars bars (60 g)	chocolate bar				
Chocolate wafer biscuits (eg. Kit-kat, Twix)	Kit-Kat size				
Chocolate bar with marzipan jelly and nougat filling	chocolate bar				
Chocolate (“New Energy”)	chocolate bar				
Sweets (eg. marshmallows, jelly, fudge, boiled sweets)	pcs.				
Others – describe type and amount:					

Snacks

	Number	Morning	Midday	Afternoon	Evening
Crisps (1 handful = 8 flakes)	handful				
Crisps low fat (1 handful = 8 flakes)	handful				
Cheese doodles (1 handful = 8 doodles)	handful				
Peanuts	bag (100 g)				
Dip (fx sour cream, cheese dip)	soup spoon				
Others – describe type and amount:					

THE EBBA SURVEY

(Breast cancer and lifestyle)

English translation; Mrs. Anne Clancy

Logbook (diary) for recording saliva samples and physical activity

Instructions for filling in the logbook

Fill in the logbook daily Serial no.__________

-DATE Write down day, date, month and year, e.g.: Tuesday 16th October 2001

-SLEEP Write down the number of hours sleep you had in the last 24 hrs.

-TIME FOR SAMPLE indicates the time you took the saliva sample.
Use a 24-hour clock, e.g.: 07.30 for morning and 19.30 for the evening. If you happen to miss out on a sample, write, "missing".
The more accurately you record date and time for sample, the easier it will be to identify your samples reliably at a later date.

-MENSTRUAL BLEEDING points to menstruation during the past 24 hrs.
Answer yes or no.

-TYPE AND DURATION OF ACTIVITY
We wish to know how you got to and from work, the shops, leisure time activities etc. during the day. Fill in the means and duration of the transport you used.

-At work:
We wish to know all types of activities you took part in during your day at work. Choose the level of activity you think suits best for each work task performed. Fill in the duration of the activity.

-At home, indoors and outdoors:
We wish to know all the activities you were engaged in, other than those you have mentioned at work and at home. Choose the level of activity that suits best for each task performed. Fill in the duration of the activity. In addition, you can mention what the task was.

-Leisure time
We wish to know all types of activities you were engaged in, in addition to those at home and at work. Choose from the list of activities, or write down in your own words the activities you took part in during the day. Use the intensity scale from 1-4 to describe how much you exerted yourself during each activity. Remember to write down the duration of the activity.

-Additional information
It is possible for you to write comments here and if necessary other remarks that you did not have room for in the section on physical activity.

Day 1	Sample collection		
Date Day of the week	Sleep (no. of hours)	Time you woke up (use 24 hr. clock)	Menstrual bleeding (Yes/No)

Additional information:

Different types of leisure time activity

1. Reading books/watching TV
2. Taking a walk/walking with a pram a pra
3. Hiking in the forest/mountains
4. Picking berries/mushrooms
5. Jogging/running
6. Bicycling
7. Exercising/gymnastics/aerobics
8. Downhill skiing/telemark/snowboard
9. Cross country skiing
10. Tennis, badminton
11. Golf/bowling
12. Weight-lifting/body strengthening
13. Swimming
14. Skating
15. Other

Level of intensity

1 =Mainly sedentary
2 = Light exercise. You do not perspire and your heart does not beat faster.
3 = Moderate exercise. You perspire a little and your heart beats a little faster.
4 = Heavy exercise: You perspire profusely and your heart beats fast

Physical activity		
Type of activity		Duration (hrs/mins)
Transport	Car	
	Bus/train/tram	
	Bicycle	
	Walking	
	Jogging/running	
Work	Sitting still	
	Standing	
	Walking at a slow pace	
	Light manual work	
	Heavy manual work	
At home in the house and outdoors	Sitting down (sewing)	
	Standing (preparing food)	
	Walking at a slow pace (dusting)	
	Moderately heavy work (vacuum cleaning)	
	Heavy work (washing floors)	

Leisure time	Type of activity	Duration (hrs/mins)	Intensity (1-4)

Appendix B

- Spørreskjema generelt
- Livskalender
- Spørreskjema p-piller
- Fotobok, kodebok og spørreskjema kost
- Instruksjon og logbok for fysisk aktivitet og spyttprøver

EBBA-STUDIEN

Sammenhengen mellom livsstil og brystkreft

Vi vet lite om de direkte årsakene til de ulike kreftsykdommene. Av den grunn er det uvisst hva hver enkelt av oss selv kan gjøre for å beskytte seg mot kreft. Hovedformålet med denne undersøkelsen er å skaffe ny kunnskap om disse sykdommene for å kunne forebygge dem. Vi ber deg svare på spørsmål om levevanene dine og helsen din. Din innsats vil være et viktig bidrag til god og praktisk anvendelig kunnskap om hvordan vi kan forebygge disse alvorlige sykdommene. Undersøkelsen er tilrådd av Regional komité for medisinsk forskningsetikk.

Svarene du gir behandles strengt fortrolig og brukes bare til forskning. Opplysningene kan senere bli sammenholdt med informasjon fra andre offentlige helseregistre etter de regler som Datatilsynet og Regional komite for medisinsk forskningsetikk gir.

På forhånd takk for hjelpen!

Med vennlig hilsen
Inger Thune dr.med.

KONFIDENSIELT

GENERELLE OPPLYSNINGER

Fødekommune ______________________

(Hvis du er født utenfor Norge, oppgi land i stedet)

Sivilstand (Sett kryss i den ruten som passer best)

- Enslig .... ☐
- Gift/samboer .... ☐
- Enke .... ☐
- Separert/skilt .... ☐
- Annet .... ☐

Hvor mange års skolegang har du i alt?
(F.o.m. folkeskole/grunnskole/yrkesutdanning/høgskole/-universitet) ______ år

Hvor mange år har du i yrkesaktiv alder hovedsakelig vært

- Hjemmearbeidende (inkl. sv. skapsperm.)? ______ år
- Heltidsarbeidende utenfor hjemmet? ______ år
- Deltidsarbeidende utenfor hjemmet? ______ år

	Ja	Nei
Har du søsken?	☐	☐

Hvis **Ja**, hvor mange . . . .Søstre? .... ________
Brødre? .... ________

Hvor mange barn hadde moren din født før du ble født? .... ________

Hvilken etnisk tilhørighet har dine forfedre?
(Foreldre/besteforeldre) (Sett kryss i de rutene som passer best)

- Norsk .... ☐
- Samisk .... ☐
- Annen europeisk .... ☐
- Finsk .... ☐
- Asiatisk .... ☐
- Annet; spesifiser ______

HØYDE/VEKT

Det kan være vanskelig å kjenne til høyde og vekt fra oppvekst og senere i livet. Likevel ber vi deg forsøke.

Fødsel:	Vekt ______ gram	Høyde ______ cm
18 år:	Vekt ______ kg	Høyde ______ cm
Dagens:	Vekt ______ kg	Høyde ______ cm

Hvordan mener du kroppen din var i forhold til jevnaldrende i oppveksten? (Sett ett kryss i hver aldersgruppe)

	Mye tynnere	Tynnere	Normal	Tykkere	Mye tykkere
Førskolealder	☐	☐	☐	☐	☐
1.-6. klasse	☐	☐	☐	☐	☐
7.-9. klasse (13-16 år)	☐	☐	☐	☐	☐

MENSTRUASJON/SVANGERSKAP/AMMING

Hvor gammel var du da du fikk din første menstruasjon? ______år ______måneder

Hvor lang tid tok det før menstruasjonen ble regelmessig? (Sett ett kryss i den ruten som passer best)

- Ett år eller mindre .... ☐
- Mer enn ett år .... ☐
- Aldri .... ☐
- Husker ikke .... ☐

Hvordan har menstruasjonen din vært ?
(Sett ett kryss)

- Alltid regelmessig .... ☐
- Oftest regelmessig .... ☐
- Uregelmessig .... ☐

Hva er gjennomsnittlig antall dager mellom hver menstruasjon? (fra 1. dag i en menstruasjon til 1. dag i neste menstruasjon) ________dager

	Ja	Nei
Har du født barn?	☐	☐
Hvis Ja, har du noen gang fått legebehandling for kvalme i svangerskap?	☐	☐

Dersom du har født barn, fyll ut fødselsår og antall måneder du ammet hvert barn (fylles også ut for barn som er død senere i livet eller ved fødselen).

Barn nr	Fødselsår	Antall måneder med amming

FYSISK AKTIVITET

HUSARBEID

SISTE ÅRET

Tenk deg en gjennomsnittlig uke med husarbeid (arbeid i hjemmet, stell/påkledning av barn/pleietrengende) det siste året. Vi har delt husarbeid inn i 4 aktivitetsnivåer. Fyll inn for hvert aktivitetsnivå **antall dager pr uke** og gjennomsnittlig **tid pr dag (minutter)** du utførte arbeidet.

Aktivitetsnivå for husarbeid er definert som:	Dager pr uke	Min. pr dag
1 = **Overveiende stillesittende arbeid;** (f.eks. sy-,skrivearbeid)		
2 = **Lett arbeid, stående, sittende,** i sakte gange; (f.eks. matlaging, støvtørk)		
3 = **Middels tungt arbeid. Du svetter litt og hjertet slår litt fortere;** (f.eks. klesvask, støvsuging, stell/påkledning)		
4 = **Tungt arbeid. Du svetter mye og hjertet slår fort;** (f.eks. tung husvask)		

GJENNOM LIVET

Husarbeid endrer seg med livssituasjon og alder. Ved hjelp av de samme aktivitetsnivåer som over (1-4) ber vi deg i ulike aldre notere ned; **gjennomsnittlig antall dager pr uke og timer pr dag** hvert aktivitetsnivå ble utført.

Aktivitetsnivå:		Alder			
		10 -14 år	15-19 år	20-24 år	25-29 år
1 = Stille-sittende arbeid	Dager pr uke				
	Timer pr dag				
2 = Lett arbeid	Dager pr uke				
	Timer pr dag				
3 = Middels tungt arbeid	Dager pr uke				
	Timer pr dag				
4 = Tungt arbeid	Dager pr uke				
	Timer pr dag				

FRITIDSAKTIVITET (MOSJON, TURGÅING, IDRETT)

SISTE ÅRET

Hva slags aktivitet har du utført i din fritid det siste året? Dersom aktiviteten varierer mye, f.eks. mellom sommer og vinter, så tenk deg et gjennomsnitt. (Sett ett kryss i den ruten som passer best.)

1= Leser, ser på fjernsyn eller annen stillesittende aktivitet . . . . ☐

2= Spaserer, sykler eller beveger deg på annen måte minst fire timer i uken (Her skal du også regne med gang eller sykling til arbeidssted, søndagsturer m.m.) . . . . . . ☐

3= Driver mosjonsidrett, tyngre hagearbeid e.l. (Merk at aktiviteten skal vare i **minst fire timer** i uken.) . . . . ☐

4= Trener hardt eller driver konkurranse idrett regelmessig og flere ganger i uken. . . . . ☐

FRITIDS AKTIVITETER OG KODER

Nedenfor ser du en liste med ulike fritidsaktiviteter. Vi ber deg finne de aktiviteter du har deltatt i. Oppgi i skjemaet nedenfor hvor lang tid (antall ganger pr måned og tid pr gang) du deltok i de ulike aktivitetene. Kryss av for det aktivitets-nivå som passer best for hver aktivitet ved hjelp av de 4 nivåer for fritidsaktivitet som er oppgitt nedenfor i aktivitetsnivå for fritidsaktiviteter.

1. Lese bøker/TV-titting
2. Gå til/ fra arbeid/ skole/ spaserturer/ trille barnevogn.
3. Gå i skogen/på fjellet/jakte
4. Jogge/løpe
5. Sykle til/fra arbeid/skole (også trimsykkel)
6. Svømme (også dykke/stupe)
7. Håndball/basketball/slåball/ fotball
8. Volleyball
9. Tennis/badminton/squash
10. Golf/bowling/curling
11. Friidrett kast/ høyde-lengde
12. Gymnastikk/aerobic/trim turning/dans/ballett
13. Styrketrening/vekttrening
14. Hoppe tau/strikk
15. Ski; langrenn - turgåing/ konkurranse
16. Kjøre slalåm/telemark/ snowboard
17. Ake/sparke/ gå på skøyter (også rulleskøyter)
18. Hesteridning
19. Ro/padle/seile
20. Plukke bær/sopp/fiske
21. Annet

AKTIVITETSNIVÅ FOR FRITIDSAKTIVITETER:

1 = Overveiende stillesittende.
2 = Lett trening. Du blir ikke svett og hjertet slår ikke fortere.
3 = Middels hard trening. Du svetter litt, og hjertet slår litt fortere.
4 = Hard trening. Du svetter mye, og hjertet slår fort.

Aktivitet Aktivitetstype	Mnd pr år	Gj.sn. ant. ggr. pr mnd.	Gj.sn. tid pr gang (min)	Aktivitetsnivå			
				1	2	3	4

GJENNOM LIVET

Se igjen på listen over de ulike fritidsaktiviteter. Vi ber deg finne de aktiviteter du har deltatt i. Deretter angi alder ved ufført aktivitet og tiden du deltok (måneder pr år, tid pr uke og hver gang). Sett **ett** kryss for det aktivitetsnivået som passer best for hver aktivitet ved hjelp av de 4 nivåene oppgitt for fritidsaktivitet ovenfor.

Aktivitet og kode	Alder start	Alder slutt	Mnd pr år	Timer pr uke	Gj.sn tid pr gang (min)	Aktivitets-nivå			
						1	2	3	4

TV-TITTING, STILLESITTING, HVILE I FRITIDEN.

Hvor mange minutter eller timer av fritiden din pr døgn bruker du gjennomsnittelig på følgende aktiviteter? Ta et gjennomsnitt for siste året.

	Antall timer	Antall minutter
Hvile, sove	______	______
Lytte til musikk/radio	______	______
TV-/videotitting	______	______
Måltid, kaffe/te	______	______
Lese, skrive	______	______
Samtale, også pr telefon	______	______
Håndarbeid, hobby	______	______

ARBEIDS/SKOLE AKTIVITETER

SISTE ÅRET

Har du vært yrkesaktiv/student de siste 12 mnd?

Ja ☐ Nei ☐

Hvis Ja; Antall

Arbeidsmåneder siste 12 mnd: . . . ________ mnd
Arbeidsdager pr uke: . . . . ________ dager
Arbeidstimer pr dag: . . . . ________ timer

Hva slags aktivitet har du vanligvis i arbeidet ditt / på skolen nå? (Sett **ett** kryss i den ruten som passer best)

1= For det meste stillesittende arbeid
(f.eks. kontorarbeid) . . . . ☐

2= Arbeid som krever at du går mye
Du blir ikke svett og hjertet slår ikke fortere
(f.eks. ekspeditør, lærer, frisør) . . . . ☐

3= Arbeid der du går og løfter mye.
Du svetter litt og hjertet kan slå raskere
(f.eks.syke-/hjelpepleier, postbetjent) . . . . ☐

4= Tungt kroppsarbeid.
Du svetter en del og hjertet slår raskt
(f.eks. tunge løft, landbruk, tungt omsorgsarbeid) . . . . ☐

Tenk deg en gjennomsnittlig uke med aktiviteter i arbeidet/på skolen i det siste året. Vi har også her delt aktivitetene inn i de samme 4 aktivitetsnivåer. Fyll inn for hvert aktivitetsnivå antall måneder pr år, timer pr uke og timer gjennomsnitt pr dag du utførte aktiviteten.

Type arbeids aktivitetsnivå	Måneder pr år	Timer pr uke	Gjennom-snitt pr dag
1= Stillesittende			
2= Står og går			
3= Går og bærer			
4= Tungt			

TRANSPORT TIL/FRA ARBEID/SKOLE

Spørsmålene gjelder transport mellom hjemmet og arbeidsplassen det siste året.

Hvordan kommer du deg vanligvis til/fra arbeid?

Ta et gjennomsnitt for en måned (Angi antall ggr du bruker de forskjellige transportmidlene.) Antall ggr. pr mnd

1.Bil . . . . ________
2.Buss/trikk/tog/båt . . . . ________
3.Sykkel . . . . ________
4.Til fots . . . . ________

Hvor lang tid tar vanligvis transporten til/fra arbeid?

Legg sammen tiden tur/retur for hvert transportmiddel. Oppgi «til fots» tiden du eventuelt går til/fra p-plass/ busstopp e.l.

1.Bil . . . . ________min
2.Buss/trikk/tog/båt . . . . ________min
3.Sykkel . . . . ________min
4.Til fots . . . . ________min

GJENNOM LIVET

Vi ber deg først skrive hvilke skoler/yrker du har/har hatt. Dersom du har hatt arbeidsplass i hjemmet som annet enn husmor, f.eks. gårdbruker, dagmamma, sydame, oppgi dette som yrker. Du kan ha hatt flere yrker i samme tidsrom, f.eks. ved deltidsarbeid. Fyll inn når du startet og sluttet i hvert yrke og hvor mye tid (antall måneder pr år, dager pr uke og timer pr dag) du vanligvis bruker/brukte. Kryss av for det aktivitetsnivået som passer best for hvert yrke ved hjelp av de 4 nivåene over.

Yrker/ skoler	Alder start	Alder slutt	Mnd pr år	Dager pr uke	Timer pr dag	Aktivitetsnivå			
						1	2	3	4

LEGEMIDLER

Vi ber deg krysse **Ja** for legemiddler du har brukt (uansett mengde) av og til og **Nei** for dem som du aldri har brukt. Dersom du krysser **Ja**, forsøk å huske alderen din første gangen du tok legemiddelet og antall ggr. pr måned du bruker det nå.

	Ja	Nei	Alder 1. gang	Ant. ggr. pr mnd
Blodtrykksmedisin............	❑	❑	______	______
Smertestillende................. (Acetylsalesylsyre/Albyl E)	❑	❑	______	______
Midler mot depresjon........	❑	❑	______	______
Hvis Ja, hvilke ______________				
Andre	❑	❑	______	______
Hvis Ja, hvilke ______________				

Vi ber deg krysse Ja for følgende legemidler du bruker fast (daglig, nesten daglig):

	Ja
Sovemedisin	❑
Smertestillende	❑
Blodtrykksmedisin	❑
Medisin mot depresjon	❑
Andre legemidler	❑
Hvis Ja, hvilke ______________________	
Naturmedisin	❑
Hvis Ja, hvilke ______________________	

KREFT I FAMILIEN

	Ja	Nei
Har nære biologiske slektninger av deg hatt kreft?	❑	❑

Hvis Ja, hvilken type kreft på mors- og farside;

Mors side	*Type kreft*	Nei	Vet ikke	*Fars side*	*Type kreft*	Nei	Vet ikke
Mor	________	❑	❑	Far	________	❑	❑
Mormor	________	❑	❑	Farfar	________	❑	❑
Morfar	________	❑	❑	Farmor	________	❑	❑
Tante	________	❑	❑	Onkel	________	❑	❑
Onkel	________	❑	❑	Tante	________	❑	❑
Andre	________	❑	❑	Andre	________	❑	❑
angi slektsskap ________				angi slektsskap ________			

LEVEVANER

	Ja	Nei
Har du noen gang røykt daglig?	❑	❑

Hvis Ja, hvor mange sigaretter røykte du gjennomsnittlig daglig? (Sett ett kryss i de ulike aldersgruppene)

Antall sigaretter daglig

	0	1-4	5-9	10-14	15-19	20-24	25+
12-14 år	❑	❑	❑	❑	❑	❑	❑
15-19 år	❑	❑	❑	❑	❑	❑	❑
20-24 år	❑	❑	❑	❑	❑	❑	❑
25-34 år	❑	❑	❑	❑	❑	❑	❑

	Ja	Nei
Røyker du daglig nå?	❑	❑

Hvis Ja, hvor mange sigaretter pr dag? ______stk

Hvor mange dagligrøykere bodde du sammen med i følgende aldre? (Sett ett kryss på hver linje.)

Antall personer:	Ingen	1	2	3 el. flere	Vet ikke
Barneårene	❑	❑	❑	❑	❑
15-19 år	❑	❑	❑	❑	❑
20-24 år	❑	❑	❑	❑	❑
25-34 år	❑	❑	❑	❑	❑

	Ja	Nei
Bor du sammen med noen som røyker nå?	❑	❑

Hvis Ja, hvor mange sigaretter røyker de i gjennomsnitt sammenlagt daglig når du er sammen med de? ____________stk

	Ja	Nei
Har du noen gang arbeidet på røykfulle arbeidsplasser?	❑	❑

Hvis Ja, hvor lenge til sammen? _________ antall år

	Ja	Nei
Har du noen gang drukket alkohol?	❑	❑

Hvis Ja, hvor mange glass vin, 1/2 litere øl eller drinker brennevin drakk du i gjennomsnitt pr måned i følgende aldre? (Sett ett kryss på hver linje)

	aldri/ sjelden	1 pr mnd.	2-3 pr mnd.	1 pr uke	2-4 pr uke	5-6 pr uke	1+ pr dag
15-19 år	❑	❑	❑	❑	❑	❑	❑
20-24 år	❑	❑	❑	❑	❑	❑	❑
25-34 år	❑	❑	❑	❑	❑	❑	❑

	Ja	Nei
Er du totalavholdskvinne nå?	❑	❑

Hvis Nei, hvor mange glass vin, 1/2 litere øl eller drinker brennevin drakk du i gjennomsnitt pr måned eller pr uke siste året? (Sett ett kryss på hver linje).

	aldri/ sjelden	1 pr mnd.	2-3 pr mnd.	1 pr uke	2-4 pr uke	5-6 pr uke	1+ pr dag
Øl (1/2l)	❑	❑	❑	❑	❑	❑	❑
Vin (glass)	❑	❑	❑	❑	❑	❑	❑
Hetvin (0,4 dl)	❑	❑	❑	❑	❑	❑	❑
Brennevin (drinker)	❑	❑	❑	❑	❑	❑	❑

Dine kommentarer

____

____

____

Vi ber om tillatelse til å kontakte deg på nytt ved et senere tidspunkt for å oppdatere opplysningene.

Ja ❑ Nei ❑

Takk for at du ville delta i undersøkelsen!

Livskalender

Det kan være vanskelig å huske hva som skjedde, hvor fysisk aktiv man har vært, hva man drev på med i forskjellige perioder av livet. Kanskje kan det hjelpe å fylle ut en slik livskalender før du begynner å svare på spørreskjemaet.

År	Hva skjedde?
1964	
1965	
1966	
1967	
1968	
1969	
1970	
1971	
1072	
1973	
1974	
1975	
1976	
1977	
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1992	
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1995	
1996	
1997	
1998	
1999	
2000	
2001	
2002	

Forslag til hendelser du kan skrive inn:

- Fødselsår
- Start barneskole
- Start ungdomsskole
- Første menstruasjon
- Start evt andre skoler
- Arbeid
- Fødsel evt barn
-

P-PILLER/ P-SPRØYTE/ HORMONSPIRAL **Løpenr __________**

	Yes	No
Har du noen gang brukt p-piller, minipiller inkludert?	☐	☐
Har du noen gang brukt p-sprøyte?	☐	☐
Har du brukt hormonspiral?	☐	☐

Hvis du har født barn, brukte du p-piller/ sprøyte/ spiral før første fødsel?	☐	☐
Har du fått p-piller/sprøyte/spiral av andre årsaker enn prevensjon?	☐	☐
Har du blitt anbefalt å slutte med p-piller/ sprøyte/spiral Av medisinske årsaker	☐	☐

Vi ønsker mer detaljert informasjon om p-piller/sprøyte/spiral bruk.
Kan du huske hvilke perioder du har brukt p-piller/sprøyte/spiral sammenhengende?
Hvor gammel var du da du startet?
Hvor gammel var du da du sluttet?
Hvor lenge brukte du det samme p-piller/sprøyte/spiral market=
Hva var navnet på p-piller/sprøyte/spiral (se vedlagt liste over navn og nummer);
Dersom du ikke husker merket, skriv 'usikker' i nevnefeltet?

Periode	Alder start	Alder slutt	Semmenhengende År	Måneder	P-pille Nummer	Navn
Første						
Andre						
Tredje						
Fjerde						
Femte						
Sjette						
Syvende						

P-piller/sprøyte/spiral merker:

Enfase-piller

Vanlig bruk: 1 tabelett daglig i 21-22 dager, deretter opphold (evnt placebotabelett i 6-7 dager.

(1) **Follimin**

(2) **Microgynon**

(3) **Eugynon**

(4) **Marvelon**

(5) **Yasmin**

(6) **Diane**

(7) **Loette**

Sekvens-piller

Vanlig bruk: Leveres i datopakninger

(8) **Synfase**

(9) **Trinordiol**

(10) **Trionetta**

Minipiller

(11) **Conludag**

(12) **Exlutona**

(13) **Microluton**

P-sprøyte

(14) **Depo-provera**

Hormonspiral

(15) **Levonova**

Annet

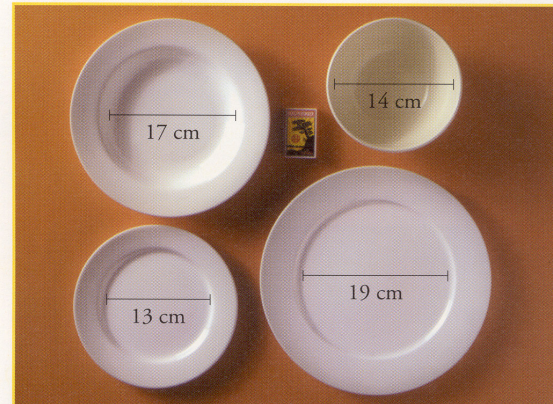
(16) Name the brand

Usikker

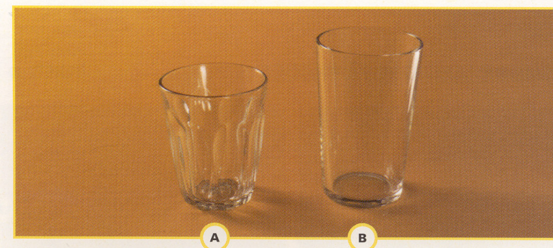
(17)

Bildehefte
med porsjonsstørrelser

DETTE BILDET VISER STØRRELSEN PÅ TALLERKENENE SOM ER BRUKT I BILDEHEFTET

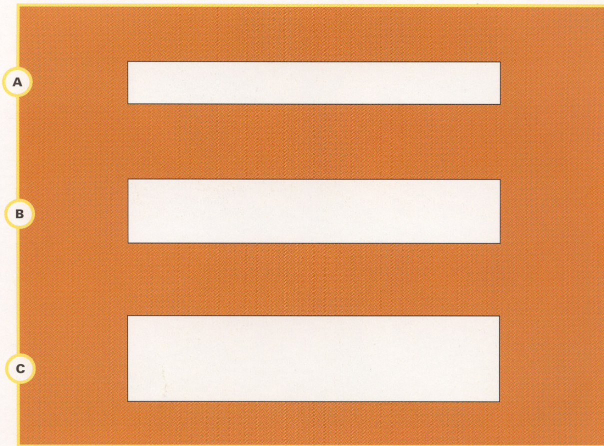


1. GLASS

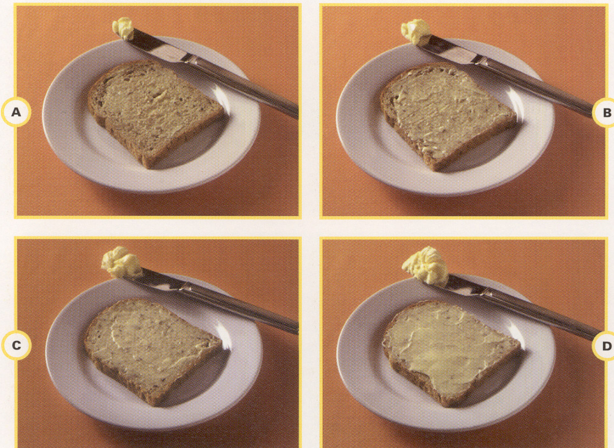


2. KOPPER

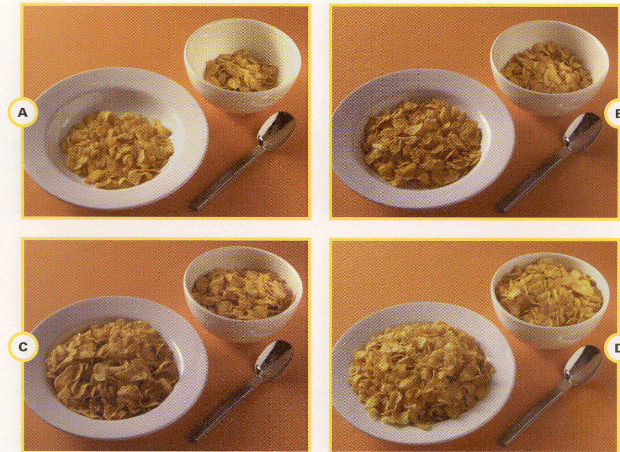
3. BRØD TYKKELSE



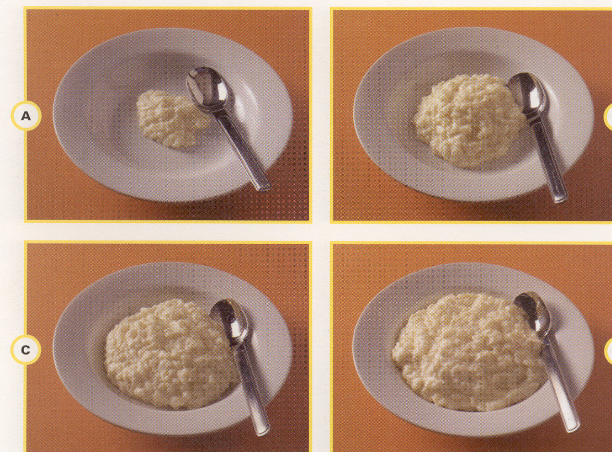
4. SMØR/MARGARIN PÅ BRØDET



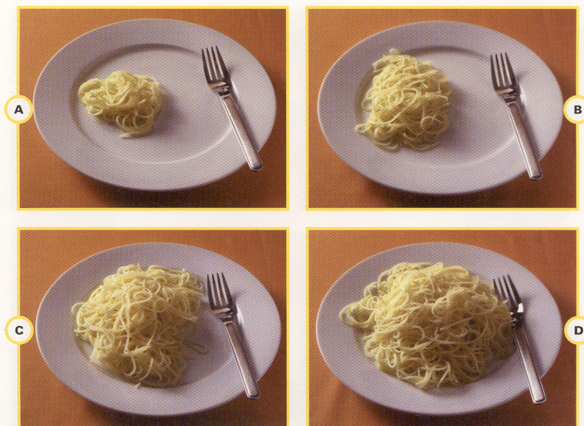
5. CORNFLAKES (FROKOSTBLANDING)



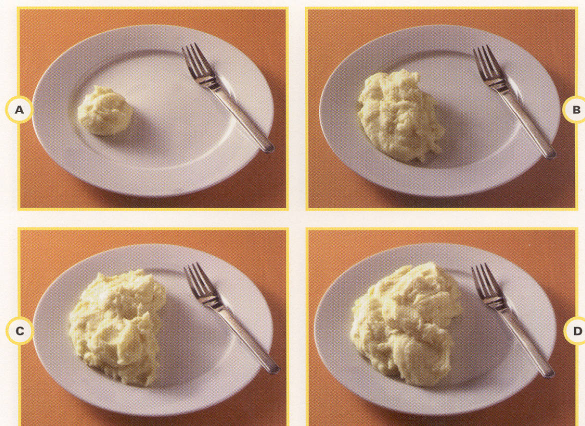
6. GRØT



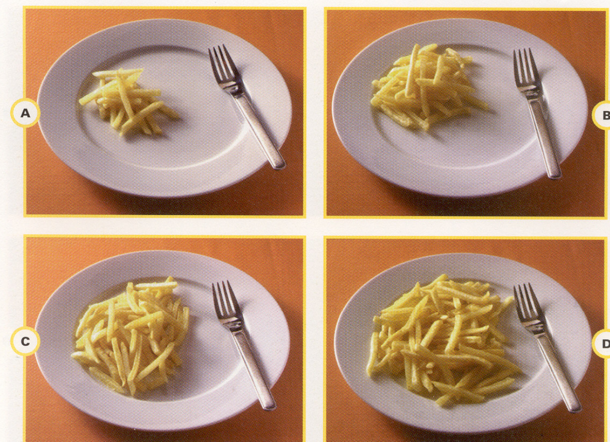
7. SPAGHETTI / PASTA (RIS)



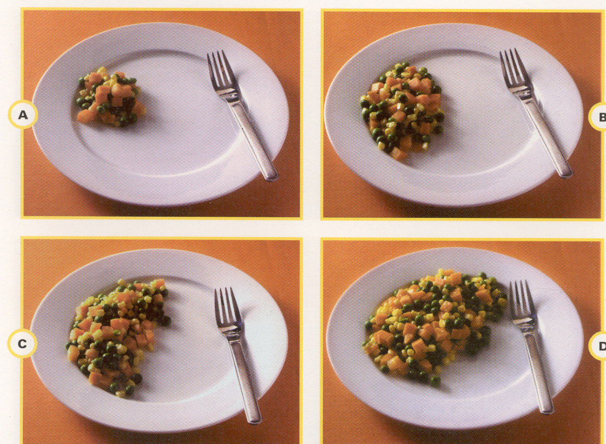
8. POTETMOS



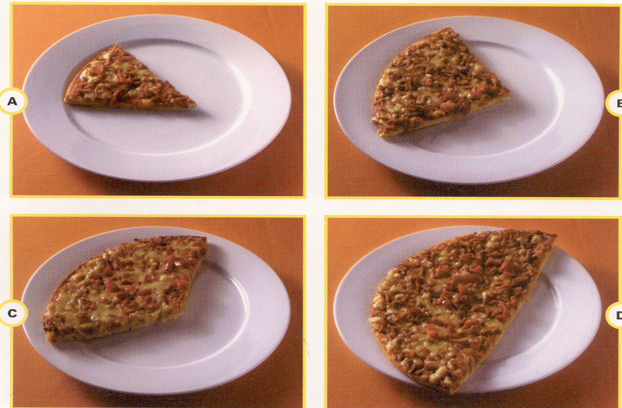
9. POMMES FRITES (STEKT POTET)



10. GRØNNSAKSBLANDING (RÅKOST)

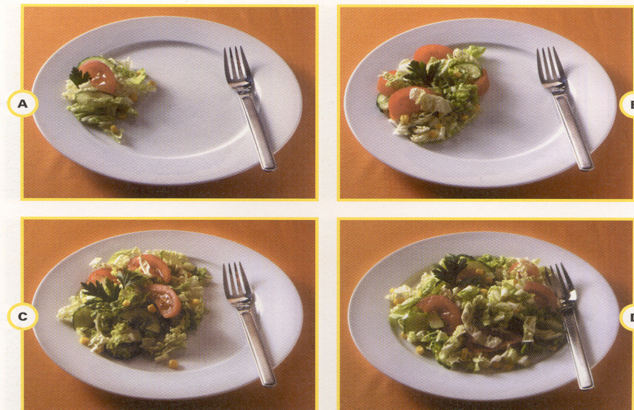


13. PIZZA, TREKANTSTYKKER

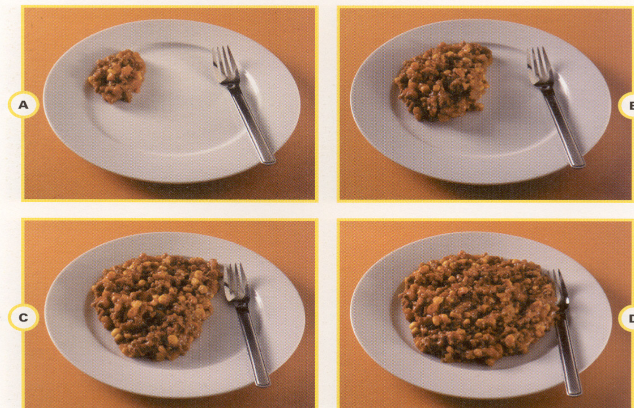


14. PIZZA, FIRKANTSTYKKER

11. SALAT



12. KJØTTSAUS (LAPSKAUS)



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STATENS RÅD FOR ERNÆRING OG FYSISK AKTIVITET

SNT Statens næringsmiddeltilsyn

INSTITUTT FOR ERNÆRINGSFORSKNING

EBBA-STUDIEN

Sammenhengen mellom livsstil og brystkreft

Den Norske Kreftforening

Kostdagbok

Dato: .... Ukedag: .... Reg. dag: ....

Var denne dagen en ganske vanlig dag eller en helt uvanlig dag med hensyn til hva du spiste og drakk?

Vanlig dag ❑ Uvanlig dag ❑

Hvis uvanlig dag angi årsak:

....

Hvor finner jeg de forskjellige matvarene?

Drikke	side 2	Poteter/ris/pasta	side 4
Yoghurt	side 2	Grønnsaker	side 4
Brød	side 2	Sauser/dressinger	side 4-5
Frokostgryn/grøt	side 2-3	Is/dessert	side 5
Pålegg	side 3	Frukt/bær	side 5
Kjøtt og kjøttretter	side 3-4	Kaker/kjeks	side 5
Fisk og fiskeretter	side 4	Sjokolade/godterier	side 5
Annen varm mat/salater	side 4	Snacks	side 5

Tran/kosttilskudd

1 barneskje = 5 ml	Antall		Morgen	Formiddag	Ettermiddag	Kveld
Tran		barneskje				
Trankapsler		stk				
Sanasol		barneskje				
Biovit		barneskje				
Multivitaminpille (eks. Vitaplex, Vitamineral)		stk				
Fluortabletter		stk				
Jerntabletter (9mg)		stk				
C-vitaminer (eks. Ester C)		stk				
Annet – beskriv type og mengde:						

Drikke

Bruk **Bildeserie 1 og 2** for å angi størrelsen på glassene og koppene

1/2 liter = 2,5 glass

	Antall	Morgen	For-middag	Etter middag	Kveld
Vann, usøtet mineralvann (eks. Farris)	glass				
Helmelk (søt/sur)	glass				
Lettmelk (søt/sur)	glass				
Ekstra Lett lettmelk	glass				
Skummet melk (søt/sur)	glass				
Drikkeyoghurt	glass				
Sjokolademelk (eks. O'Boy, Litago)	glass				
Kakao	kopp				
Juice, nektar	glass				
Brus, saft med sukker	glass				
Brus, saft uten sukker	glass				
Te	kopp				
Iste med sukker	glass				
Kaffe	kopp				
Suketter (eks. Natrena, Canderel)	stk				
Sukker til te, kaffe	teskje				
Melk til te, kaffe	spiseskje				
Øl	1/2 liter				
Vin	vinglass				
Brennevin	drink				
Annet – beskriv type og mengde:					

Yoghurt

	Antall	Morgen	For-middag	Etter middag	Kveld
Yoghurt naturell	beger (175 ml)				
Yoghurt med frukt	beger (175 ml)				
Lettyoghurt	beger (150 ml)				
Go'morgen yoghurt m/müsli	beger (inkludert müsli)				
Annet – beskriv type og mengde:					

Brød m.m.

Bruk **Bildeserie 3** for å angi tykkelse på brødet

1 skive = 1/2 rundstykke

	Antall	Morgen	For-middag	Etter middag	Kveld
Loff, fint rundstykke	skiver bildeserie 3				
Mellomgrovt brød (eks. kneip),- grovt rundstykke	skiver bildeserie 3				
Grovbrød	skiver bildeserie 3				
Baguette, ciabatta	stk				
Knekkebrød	stk				
Lompe	stk				
Pølsebrød, hamburgerbrød	stk				
Flatbrød	stk				
Annet – beskriv type og mengde:					

Hva smurte du på brødet?

Angi hvor mye smør/margarin du har på brødet, se **bildeserie 4**

1 skive = ½ rundstykke = 2 kjeks

	Antall	Morgen	For-middag	Etter middag	Kveld
Meierismør	skiver				
Myk margarin (eks. Soya soft)	skiver				
Lett margarin (eks. Soft light)	skiver				
Hard margarin (eks. Per, Melange)	skiver				
Annet – beskriv type og mengde:	skiver				

Frokostgryn og grøt

	Antall	Morgen	For-middag	Etter middag	Kveld
Havregrøt	bildeserie 6				
Havregryn, firkorn	bildeserie 5				
Müsli søtet (eks. Crüsli, Solfrokost)	bildeserie 5				
Müsli usøtet (eks. Go'Dag, Gullfrokost)	bildeserie 5				
Cornflakes	bildeserie 5				
Frosties, chocofrokost, honnikorn	bildeserie 5				
Annet – beskriv type og mengde:					

Tilbehør til frokostgryn og grøt

1 spiseskje = 3 teskjeer

	Antall	Morgen	For-middag	Etter middag	Kveld
Helmelk (søt/sur)	dl				
Lettmelk (søt/sur)	dl				
Ekstra lett lettmelk	dl				
Skummet melk (søt/sur)	dl				
Syltetøy vanlig, gelé, marmelade	teskjeer				
Syltetøy lett, frysetøy	teskjeer				
Sukker	teskjeer				
Annet – beskriv type og mengde:					

Pålegg

Oppgi mengde pålegg i forhold til brødskiver.
Om du har spist to typer pålegg på samme brødskive, fører du opp begge. (Eks. 1 hvitost helfet og 1 skinke.)
Hvis du bare har spist pålegg og ikke brød, anslå til hvor mange skiver du kunne brukt dette pålegget.

1 skive = 1/2 rundstykke = 1 knekkebrød = 2 kjeks

	Antall	Morgen	For-middag	Etter middag	Kveld
Ost					
Hvitost helfet 27% fett (eks. Jarlsberg, Norvegia)	til antall skiver				
Hvitost halvfet 16% fett (eks.Norvegia lettere)	til antall skiver				
Brunost helfet (eks. Geitost G35, Fløtemysost)	til antall skiver				
Brunost halvfet, prim	til antall skiver				
Smøreost, vanlig (eks. Baconost, Snøfrisk)	til antall skiver				
Smøreost, mager (eks. mager skinkeost, mager prim)	til antall skiver				
Dessertoster (eks. Brie, Gräddost, Gourmet frukt)	til antall skiver				
Kjøttpålegg					
Servelat vanlig	til antall skiver				
Skinke, spekeskinke, lettservelat	til antall skiver				
Salami, spekepølse, fårepølse	til antall skiver				
Leverpostei vanlig	til antall skiver				
Leverpostei mager	til antall skiver				
Fiskepålegg					
Kaviar	til antall skiver				
Røkt laks, ørret	til antall skiver				
Makrell i tomat, røkt makrell	til antall skiver				
Sardiner, sursild, ansjos	til antall skiver				
Syltetøy, søtt pålegg					
Syltetøy vanlig, gelé, marmelade	til antall skiver				
Syltetøy lett, frysetøy	til antall skiver				
Honning	til antall skiver				
Peanøttsmør	til antall skiver				
Sjokolade, søtt pålegg	til antall skiver				
Annet pålegg					
Majonessalat (eks. italiensk salat, rekesalat)	til antall skiver				
Majonessalat lett	til antall skiver				
Tomat som pålegg	til antall skiver				
Banan som pålegg	til antall skiver				
Majones, remulade vanlig	til antall skiver				
Majones, remulade lett	til antall skiver				
Annet – beskriv type og mengde:					

Kjøtt og kjøttretter

	Antall	Morgen	For-middag	Etter middag	Kveld
Pølse					
Grillpølse, wienerpølse vanlig	stk				
Grillpølse, wienerpølse lett	stk				
Middagspølse, kjøttpølse, medisterpølse	kjøttpølse				
Middagspølse lett, kjøttpølse lett	kjøttpølse				
Kjøttdeigretter, pasta, pizza					
Kjøttkaker, karbonadekaker	stk				
Medisterkaker	stk				
Tacoskjell (med kjøttdeig og salat)	fylte skjell				
Kebab, pitabrød (med kjøtt og salat)	fylte pitabrød				
Kjøttdeigsaus, tomatsaus med kjøttdeig	bildeserie 12				
Pasta med tomatsaus uten kjøtt	bildeserie 7				
Pasta med hvit saus	bildeserie 7				
Lasagne	stykke (10 x 5 cm)				
Pizza, firkantete stykker	bildeserie 14				
Pizza, trekantete stykker	bildeserie 13				
Rent kjøtt					
Biff (okse, lam, svin)	stk				
Koteletter (svin, lam, okse)	stk				
Stek (svin, okse, lam)	skiver				

	Antall	Morgen	For-middag	Etter middag	Kveld
Kokt skinke	skiver				
Grillet kylling	1/4 kylling				
Kyllingfilet	filèer				
Bacon	skiver				

Gryteretter

	Antall	Morgen	For-middag	Etter middag	Kveld
Risotto, risretter	bildeserie 12				
Fårikål, frikasé	bildeserie 12				
Lapskaus	bildeserie 12				
Kjøttgryte (kjøtt og grønnsaker i samme gryte)	bildeserie 12				
Leverretter	bildeserie 12				
Annet – beskriv type og mengde:					

Fisk og fiskeretter

	Antall	Morgen	For-middag	Etter middag	Kveld
Fiskefarse, fiskemat					
Fiskeboller	stk				
Fiskekaker, fiskepudding	stk/skiver				
Ren fisk					
Torsk, sei, uer (kokt)	skiver				
Torsk, sei, uer (stekt)	bildeserie 15				
Laks, ørret, kveite (kokt)	skiver				
Laks, ørret, kveite (stekt)	bildeserie 15				
Sild, makrell (kokt)	skiver				
Sild, makrell (stekt)	bildeserie 15				
Flyndre, steinbit (kokt)	skiver				
Flyndre, steinbit (stekt)	bildeserie 15				
Tillagede fiskeretter og fiskepinner					
Fiskepinner	stk				
Panert fisk	stk (10x10 cm)				
Fiskegryte, fiskesuppe	dl				
Fiskegrateng, plukkfisk	dl				
Reker uten skall	dl				
Annet – beskriv type og mengde:					

Annen varm mat/salater

	Antall	Morgen	For-middag	Etter middag	Kveld
Risengrynsgrøt	bildeserie 6				
Pannekaker	stk				
Kjøttsuppe (eks. betasuppe med kjøtt)	tallerken				
Suppe (eks. blomkålsuppe, tomatsuppe)	tallerken				
Egg, kokt, stekt	antall egg				
Omelett	antall egg				
Ostepai, quiche	stykke (10 x 8 cm)				
Blandet salat med ost, kjøtt eller skalldyr	bildeserie 11				
Salat med pasta og ost, kjøtt eller skalldyr.	bildeserie 11				
Vegetarrett – beskriv type og mengde:					
Annet – beskriv type og mengde:					

Poteter/ris/pasta

	Antall	Morgen	For-middag	Etter middag	Kveld
Potet, kokt	stk				
Potet, bakt	stk				
Potetmos	bildeserie 8				
Pommes frites	bildeserie 9				
Stekt potet	bildeserie 9				
Potetsalat	spiseskjeer				
Ris, kokt	bildeserie 7				
Pasta kokt (eks. spaghetti, makaroni, tagliatelle)	bildeserie 7				
Annet – beskriv type og mengde:					

Grønnsaker

	Antall	Morgen	For-middag	Etter middag	Kveld
Gulrot	stk				
Kålrot	skive				
Brokkoli, blomkål	dl				
Hodekål	dl				
Råkost (blandet av flere grønnsaker)	bildeserie 10				
Grønnsaksblanding kokt	bildeserie 10				
Blandet salat (eks. kinakål, mais, tomat og agurk)	bildeserie 11				
Tomat, paprika, stekt løk	ringer				
Annet – beskriv type og mengde:					

Sauser/dressinger

1 spiseskje = 3 teskjeer

	Antall	Morgen	For-middag	Etter middag	Kveld
Hvit saus	spiseskjeer				
Brun saus	spiseskjeer				
Smeltet smør, margarin	spiseskjeer				

		Morgen	For-middag	Etter middag	Kveld
Tomatsaus (uten kjøtt)	spiseskjeer				
Bernaise saus	spiseskjeer				
Dressing vanlig (eks. Thousand Island)	spiseskjeer				
Dressing lett (eks. Thousand Island light)	spiseskjeer				
Seterrømme 35 % fett	spiseskjeer				
Lettrømme 20 % fett	spiseskjeer				
Majones, remulade vanlig	spiseskjeer				
Majones, remulade lett	spiseskjeer				
Oljedressing (eks. Fransk dressing)	spiseskjeer				
Annet – beskriv type og mengde:					

Is/dessert

	Antall	Morgen	For-middag	Etter middag	Kveld
Is (eks. vanilje, krokan)	bildeserie 16				
Ispinne, kremmerhus (eks. Gullpinne, Kroneis)	stk				
Gelé	bildeserie 16				
Pudding (eks. sjokoladepudding, karamellpudding)	bildeserie 16				
Riskrem, multekrem, fromasj	bildeserie 16				
Fløte	spiseskjeer				
Krem, pisket	spiseskjeer				
Sjokoladesaus, karamellsaus	spiseskjeer				
Vaniljesaus	dl				
Annet – beskriv type og mengde:					

Frukt/bær

	Antall	Morgen	For-middag	Etter middag	Kveld
Eple, pære	stk				
Banan	stk				
Appelsin	stk				
Mandarin, klementin	stk				
Druer	stk				
Fersken, nektarin	stk				
Friske, frosne bær	dl				
Annet – beskriv type og mengde:					

Kaker/kjeks

	Antall	Morgen	For-middag	Etter middag	Kveld
Boller, kringle, skolebrød	stk				
Wienerbrød	stk				
Vafler	hjerter				
Eplekake, formkake	stykke				
Sjokoladekake	stykke				
Bløtkake	stykke				
Fyrstekake, nøttekake	stykke				
Kjeks (eks. Mariekjeks, Gjende)	stk				
Fylte kjeks (eks. Ballerina, Maryland Cookies)	stk				
Havrekjeks (eks. Bixit, Sibas)	stk				
Smørbrødkjeks (eks. Kornmo, Golden Crisp)	stk				
Smørbrødkjeks (eks. Kaptein, Start)	stk				
Salte kjeks (eks. Ritz, Salinas)	stk				
Annet – beskriv type og mengde:					

Sjokolade/godterier

	Antall	Morgen	For-middag	Etter middag	Kveld
Melkesjokolade (eks. Melkesjokolade, Firkløver, Helnøtt)	sjokoladeplate (100 g)				
Marsipan med sjokolade (eks. Gullbrød)	stk (65 gram)				
Sjokoladebiter (eks. Twist, konfekt)	biter				
Snickers, Japp (vanlig 60 g)	stk				
Kjekssjokolade (eks. Kvikklunsj,Twix)	Kvikklunsj størrelse				
Troika	stk				
New Energy	stk				
Smågodt (eks. skumgodt, gelé, lakris, karamell, vingummi, drops)	stk				
Annet – beskriv type og mengde:					

Snacks

	Antall	Morgen	For-middag	Etter middag	Kveld
Potetgull vanlig (1 neve = 8 flak)	neve				
Potetgull lett , potetskruer (1 neve = 8 flak)	neve				
Ostepop (1 neve = 8 "ostebuer")	neve				
Peanøtter	pose (100 g)				
Dip (eks. rømme m/dipmix, cheese dip)	spiseskjeer				
Annet – beskriv type og mengde:					

Takk for at du ville delta i undersøkelsen!

ID. nummer_______________

SNT Statens næringsmiddeltilsyn

EBBA-STUDIEN

Sammenhengen mellom livsstil og brystkreft

Daglig log-registrering av spyttprøver og fysisk aktivitet

INSTRUKSJONER FOR UTFYLLING **Lpnr: ______**

Fyll ut daglig log hver dag.

- **Dato** Skriv inn både dag og dato, for eksempel: tirsdag 16. Oktober 2001.
- **Søvn** Skriv inn antall timer du har sovet de siste 24 timer og tidspunkt du sto opp.

- **Tid prøve** betyr klokkeslett spytt prøver

  Bruk 24-timer angivelse. Eks: 7.30 om morgenen og 19.30 om kvelden. Dersom du mister en prøve en dag, skriv "Missing".

  Jo mer fullstendig du registrerer dato og klokkeslett for spytt prøven, jo større er sjansen for at alle dine prøver senere vil la seg identifisere korrekt.

- **Blødning** Indikerer om du har hatt menstruasjonsblødning i løpet av de siste 24 timene.

  JA dersom du har hatt blødning, NEI dersom du ikke har hatt det.

- **Aktivitetens type og varighet**

  Transport:
  Vi ønsker å vite hvordan du kom deg til og fra arbeid, butikk, fritidsarrangement etc i løpet av dagen. Velg type transport du har benyttet, og fyll inn varigheten.

  Jobb:
  Vi ønsker å vite alle typene aktiviteter du har drevet med i løpet av dagen på arbeid. Velg det nivået du synes passer best for hver arbeidsoppgave du har utført, og fyll inn varighet av aktiviteten.

  Arbeid i hjemmet inne og ute:
  Vi ønsker å vite alle arbeidsaktiviteter du har utført hjemme, enten inne eller ute, i løpet av dagen. Velg det nivået du synes passer best for hvert arbeid du har gjort, og fyll inn varighet av arbeidet. Du kan i tillegg skrive akkurat hva du har gjort.

  Fritid:
  Vi ønsker å vite alle typer aktiviteter du har drevet med utenom det du har oppgitt som arbeid i jobb eller hjemme. Velg aktiviteter fra listen eller skriv med egne ord hvilke aktiviteter du har drevet med i løpet av dagen. Bruk intensitetsskalaen 1-4 for å angi hvor mye du anstrengte deg ved hver aktivitet. Husk å angi varighet for hver aktivitetstype.

- I **tilleggsinformasjon** har du mulighet for å skrive kommentarer og evt ting du ikke får plass til i skjema for fysisk aktivitet.

Dag 3 — Prøvetaking

Dato ukedag	Søvn (ant. timer)	Tid du våknet (klokkeslett)	Tid spytt prøve (kl.)	Blødningsdag (Ja/Nei)

Tilleggsinformasjon: "Kostdagbok"

Ulike typer fritidsaktiviteter:
1. Lese bøker/TV-titting
2. Spasere/trille barnevogn
3. Gå i skogen/fjellet
4. Plukke bær/sopp
5. Jogge/løpe
6. Sykle
7. Trim/gymnastikk/aerobic
8. Slalåm/telemark/snowboard
9. Ski (langrenn, turgåing)
10. Tennis, badminton
11. Golf/bowling
12. Styrke/vekt-trening
13. Svømming
14. Ake/sparke/skøyter/rulleskøyter
15. Annet

Intensitetsnivåer for fritidsakt.:
1= Overveiende stillesittende
2= Lett aktivitet. Du blir ikke svett og hjertet slår ikke fortere
3= Middels hard aktivitet. Du svetter litt og hjertet slår litt fortere
4= Hard trening. Du svetter mye og hjertet slår fort

Fysisk aktivitet

	Type aktivitet	Varighet (t/min)
Transport	Bil	
	Buss/tog/trikk	
	Sykkel	
	Til fots: spasere	
	jogge/løpe	
Jobb	Stillesittende	
	Stående	
	Sakte gange	
	Lett kroppsarbeid	
	Hardt kroppsarbeid	
Arbeide i hjemmet inne og ute	Stillesittende (f.eks. sy)	
	Stående (f.eks. lage mat)	
	Sakte gange (f.eks. tørke støv)	
	Middels tungt (f.eks. støvsuge)	
	Tungt arbeid (f.eks. gulvvask)	

Fritid	Type aktivitet	Varighet (t/min)	Intensitet (1-4)

Dag 4 — Prøvetaking

Dato ukedag	Søvn (ant. timer)	Tid du våknet (klokkeslett)	Tid spytt prøve (kl.)	Blødningsdag (Ja/Nei)

Tilleggsinformasjon: "Kostdagbok"

Ulike typer fritidsaktiviteter:
1. Lese bøker/TV-titting
2. Spasere/trille barnevogn
3. Gå i skogen/fjellet
4. Plukke bær/sopp
5. Jogge/løpe
6. Sykle
7. Trim/gymnastikk/aerobic
8. Slalåm/telemark/snowboard
9. Ski (langrenn, turgåing)
10. Tennis, badminton
11. Golf/bowling
12. Styrke/vekt-trening
13. Svømming
14. Ake/sparke/skøyter/rulleskøyter
15. Annet

Intensitetsnivåer for fritidsakt.:
1= Overveiende stillesittende
2= Lett aktivitet. Du blir ikke svett og hjertet slår ikke fortere
3= Middels hard aktivitet. Du svetter litt og hjertet slår litt fortere
4= Hard trening. Du svetter mye og hjertet slår fort

Fysisk aktivitet

	Type aktivitet	Varighet (t/min)
Transport	Bil	
	Buss/tog/trikk	
	Sykkel	
	Til fots: spasere	
	jogge/løpe	
Jobb	Stillesittende	
	Stående	
	Sakte gange	
	Lett kroppsarbeid	
	Hardt kroppsarbeid	
Arbeide i hjemmet inne og ute	Stillesittende (f.eks. sy)	
	Stående (f.eks. lage mat)	
	Sakte gange (f.eks. tørke støv)	
	Middels tungt (f.eks. støvsuge)	
	Tungt arbeid (f.eks. gulvvask)	

Fritid	Type aktivitet	Varighet (t/min)	Intensitet (1-4)

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