Metabolic and Hormonal Profiles: HDL Cholesterol as a Plausible Biomarker of Breast Cancer Risk. The Norwegian EBBA Study

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Abstract

Low serum high-density lipoprotein cholesterol (HDL-C) is an important component of the metabolic syndrome and has recently been related to increased breast cancer risk in overweight and obese women. We therefore questioned whether serum HDL-C might be a biologically sound marker of breast cancer risk. We obtained cross-sectional data among 206 healthy women ages 25 to 35 years who participated in the Norwegian EBBA study. We included salivary ovarian steroid concentrations assessed by daily samples throughout one entire menstrual cycle, metabolic profile with measures of adiposity [body mass index (BMI) and truncal fat percentage], serum concentrations of lipids and hormones (insulin, leptin, testosterone, dehydroepiandrostendione sulfate, insulin-like growth factor-I, and its principal binding protein), and mammographic parenchymal pattern. We examined how components of the metabolic syndrome, including low serum HDL-C, were related to levels of hormones, and free estradiol concentration in particular, and studied predictors of mammographic parenchymal patterns in regression models. In women with BMI ≥ 23.6 kg/m^2 (median), overall average salivary estradiol concentration dropped by 2.4 pmol/L (0.7 pg/mL; 13.2% change in mean for the total population) by each 0.33 mmol/L (12.8 mg/dl; 1 SD) increase in serum HDL-C (P = 0.03; $P_{interaction}$ = 0.03). A subgroup of women characterized by both relatively high BMI (≥23.6 kg/m^2) and high serum LDL-C/HDL-C ratio (≥ 2.08; 75 percentile) had substantially higher levels of salivary estradiol by cycle day than other women (P = 0.001). BMI was the strongest predictor of overall average estradiol with a direct relationship (P < 0.001). Serum HDL-C was inversely related to serum leptin, insulin, and dehydroepiandrostendione sulfate (P < 0.001, P < 0.01, and P < 0.05, respectively). There was a direct relationship between breast density and healthy metabolic profiles (low BMI, high serum HDL-C; P < 0.001) and salivary progesterone concentrations (P < 0.05). Our findings support the hypothesis that low serum HDL-C might reflect an unfavorable hormonal profile with, in particular, increased levels of estrogens and gives further clues to biomarkers of breast cancer risk especially in overweight and obese women. (Cancer Epidemiol Biomarkers Prev 2005;14(1):33–40)

Introduction

The prevalence of obesity [body mass index (BMI) ≥ 30 kg/m^2] is increasing worldwide, and obese women and women with adult weight gain seem to be at increased risk of breast cancer (1). Moreover, obese women, are susceptible to the metabolic syndrome [i.e., glucose intolerance, low serum high-density lipoprotein cholesterol (HDL-C), high serum triglycerides, and hypertension], and this, in turn, seems to put women at an even higher risk of breast cancer (2-5). In a prospective study in Norway (2), we recently observed that serum HDL-C was inversely related to risk of postmenopausal breast cancer in overweight (BMI = 25-30 kg/m^2) and obese women. Thus, we hypothesized that low serum HDL-C might reflect an unfavorable hormonal profile with increased levels of breast mitogens, mainly of estrogens, but also of androgens, insulin, and insulin-like growth factor I (IGF-I; ref. 2).

The aim of the present study was to test whether the different components of the metabolic syndrome including low serum HDL-C, are associated with levels of endogenous hormones, a major physiologic link between obesity and risk of breast cancer (6) and with breast parenchymal density, a surrogate measure of breast cancer risk (7). Evidence from population-based studies might suggest reliable biomarkers of breast cancer risk among conventional clinical variables, as serum lipids, that could provide a strategy for identifying high-risk individuals who might benefit from preventative intervention. Furthermore, as metabolic profile has also been related to endometrial cancer risk in epidemiologic studies (8, 9), including a study from our group (10), learning about obesity-related biomarkers may not be limited to breast cancer.

We examined daily concentrations of estradiol and progesterone during one entire menstrual cycle using a salivary RIA, serum concentrations of lipids, glucose, insulin, leptin, androgens, SHBG, IGF-I, and IGFBP-3, and measures of adiposity (BMI and truncal fat percentage), and mammographic parenchymal patterns in 206 healthy Norwegian women ages 25 to 35 years.

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Material and Methods

Subjects and Study Design. Subjects for the present study participated in the Norwegian EBBA study.[7] A total of 206 women living in North Norway entered the study from 2000 to 2002. The women were invited to participate by announcements in newspapers and locally. Study subjects had to meet the following criteria: 25 to 35 years of age; self-reported regular cycles (cycle length, 22-38 days) within the previous 3 months; no use of hormonal contraceptives and no pregnancy or breast-feeding over the previous 6 months; no infertility, gynecologic disorders, chronic disorders (i.e., diabetes, hypothyroidism/hyperthyroidism) or abnormally high BMI (>30 kg/m^2). All clinical procedures were conducted by trained nurses at the Department of Clinical Research, University Hospital of North Norway, Tromsø. All the participating women signed an informed consent form. The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Questionnaires. We used a general questionnaire (self-administered and by interview) to collect information on ethnicity, education, reproductive history, and past and current lifestyle including physical activity (type, frequency, and duration), use of hormonal contraceptives, tobacco, and alcohol. Total average energy expenditure per week during the past year was estimated by multiplying the average number of hours per week spent at each activity by the energy cost of that particular activity expressed in metabolic rates (METs 11).

The women were asked to record the type and the portion size of every food item consumed during 24 hours in a precoded food diary developed for the EBBA study on seven selected days in the menstrual cycle (days 3-6 and days 21-23). Average daily intake of energy and nutrients were computed by using a food database and software system developed at the Institute for Nutrition Research, University of Oslo, Norway (12).

Clinical Variables and Mammogram. On the first possible day after onset of the menstrual bleeding, participants met for clinical examinations, including height, weight, and blood pressure measurement and blood collection. The majority of the women met on day 1 or day 2 of their menstrual cycle, but some women had to wait until days 3 to 5 because the medical facilities were closed during holidays and weekends. Height was measured to the nearest half centimeter and weight was measured to the nearest 0.1 kg on an electronic scale with subjects wearing light clothing and no footwear. BMI (kg/m^2) was used to estimate relative weight.

The women came in once during midcycle (days 7-12) for a whole body scan and mammography. The whole body scan was obtained by DEXA (DPX-L 2288, Lunar Radiation Co., Madison, WI) operated by one skilled nurse and percentage of fat tissue in the trunk was estimated by Lunar software. Mammograms were taken with Siemens Mammomat 3000 at the Centre of Breast Imaging, University Hospital of North Norway. The mammographic parenchymal pattern in bilateral craniocaudal and mediolateral oblique projections was categorized by radiologist N. Bjurstam using a modified Wolfe's classification (7) with four categories: I = essentially normal breast tissue with parenchyma composed primarily of fat; II = prominent ductal pattern in up to one fourth of the breast volume; III = prominent ductal pattern in more than one fourth of the breast volume; and IV = extremely dense parenchyma, which usually denotes connective tissue hyperplasia.

Serum and Saliva Samples. Blood samples were drawn after overnight fasting. Serum concentrations of glucose, triglycerides, total cholesterol, HDL-C, testosterone, DHEA-SO_4, and SHBG were measured in fresh sera at the Department of Clinical Chemistry, University Hospital of North Norway, during the study period of 26 months. Serum glucose was measured enzymatically by the hexokinase method. Serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. HDL-C was quantified by a direct assay using polyethylene glycol-modified enzymes and dextran sulfat. Serum low-density lipoprotein cholesterol (LDL-C) was estimated according to Friedewalds formula (total cholesterol − HDL-C − 0.46 × triglycerides). Serum testosterone was measured by an enhanced chemiluminescence immunoassay, serum DHEA-SO_4 was measured by a competitive immunometric assay, and serum SHBG was measured by an immunometric assay. Serum glucose and lipids were measured by kits from Roche Diagnostics GmbH, Mannheim, Germany, and serum DHEA-SO_4 and SHBG were measured by kits from Diagnostic Products Co. (DPC)-Bierman GmbH, Bad Nauheim, Germany. The serum testosterone measurement was made using Elecsys 2010 from Roche Diagnostics, in 153 samples (74%), whereas for the remaining samples Immuno 1 from Bayer Diagnostics (Tarrytown, NY) was used. The correlation coefficient was 0.97 in parallel runs of the testosterone assays and no correction formula was developed to adjust for the change of assay. Serum concentrations of insulin, leptin, IGF-I, and IGFBP-3 were measured at the Hormone Laboratory, Aker University Hospital, Oslo, in serum that were stored at −70°C for up to 3 years until analysis; all samples were assayed during a time period of 2 months. Serum insulin and leptin were measured by RIA using kits from Linco Research, Inc., St. Charles, MO. The levels of IGF-I and IGFBP-3 were determined by ILMA using Immulite 2000 from Diagnostic Products Co. (DPC), Los Angeles, CA. Apart from the described change in assay for serum testosterone, there was no marked drift in any serum variable during the study period at any of the laboratories. The coefficients of variation derived from the laboratories were as follows: 2% glucose, 2% triglycerides, 2.5% cholesterol, 3% HDL-C, <5% testosterone, <10% DHEA-SO_4, 5% to 10% SHBG, 8% to 12% insulin, 7% to 10% IGF-I, 6% IGFBP-3, and 10% to 12% leptin.

The participants collected samples of their own saliva at home once a day, preferentially in the morning, for the complete menstrual cycle. Collection of saliva followed previously established protocols (13) from the Reproductive Ecology Laboratory at Harvard University, where the saliva samples were analyzed. Estradiol was assayed in saliva samples from 20 days (reverse cycle days −5 to −24) and progesterone was assayed in saliva samples from the last 14 days of each cycle (reverse cycle days −1 to −14). Salivary estradiol measurements were made using a I-125 based RIA kit (Diagnostic Systems Laboratories, Webster, TX) with the following modifications to the manufacturer's protocol to shift the location of the standard curve: Standards were prepared in assay buffer and run in six concentrations from 0.33 to 10 pg/mL. Samples were added in 100-μL amounts together with 200 μL of assay buffer. First antibody was diluted 1:4. Labeled steroid was diluted 1:3. First antibody and labeled steroid were added to each tube in 100-μL amounts to yield a total reaction volume of 500 μL per tube. Similarly, salivary progesterone measurements were made using an I-125 based RIA kit (Diagnostic Systems Laboratories) with the following modifications: Standards were prepared in assay buffer and run at six concentrations from 2 to 200 pg/mL. Samples were added in 100-μL amounts together with 100 μL of assay buffer. First antibody was diluted 1:4. First antibody and labeled steroid were added to each tube in 100-μL amounts to yield a total reaction volume of 400 μL per tube.

[7] I Thune, et al. Body composition (height, weight, BMI, WHR, fat percentage) influences estradiol and progesterone levels throughout a menstrual cycle among women aged 25-35 years. The Norwegian EBBA-study. In preparation.

In both assays, after overnight incubation at 4°C, 500 μL of second antibody was added to each reaction tube. Reaction tubes were subsequently centrifuged for 45 minutes; after aspiration of the supernatant, tubes were counted in a gamma counter for 2 minutes. There was a maximum of 60 samples per assay (representing three or four different subjects) and subjects were randomly assigned to assays. The sensitivity of the estradiol assay is 4 pmol/L (1.1 pg/mL). Average intra-assay variability was 9%, and interassay variability ranged from 23% for low pools to 13% for high pools. For progesterone, the sensitivity of the essay is 13 pmol/L (4.1 pg/mL). Average intra-assay variability was 10%; interassay variability ranged from 19% for low pools to 12% for high pools.

Statistical Analyses. To test whether components of the metabolic syndrome including low serum HDL-C, are associated with higher levels of hormones, and free estradiol concentration in particular, and further are related to mammographic parenchymal patterns in premenopausal women, we estimated some indices of ovarian function and did a variety of statistical analyses by SAS statistical package version 8.2.

Alignment of the cycles for analysis was based on the identification of the day of the midcycle estradiol drop, which provides a good estimate of the day of ovulation (14) and was designated as ''day 0.'' Satisfactory identification of the midcycle estradiol drop could not be made for 14 women and their cycles were not aligned. Overall average salivary estradiol and progesterone were calculated for all the women, whereas a midluteal index (the average of the hormone concentrations on days + 5 to + 9) was calculated for the 192 women with aligned cycles. The midluteal index corresponds to the period of maximal progesterone secretion by the corpus luteum and a relatively high (i.e., in comparison with the follicular phase) and stable production of estradiol.

We used linear regression modeling to study the associations between measures of metabolic profile and average salivary estradiol and progesterone levels (overall and midluteal) throughout one entire menstrual cycle and adjusted for potential confounding factors in multivariate models.

We used a generalized linear model to compare average salivary estradiol concentrations by cycle day in the interval from days −10 to + 9 in subgroups of women defined by both BMI and serum lipids. To study in more detail the importance of the biological variation in serum HDL-C level, we used the 75 percentile as cut point for this categorization: group A, BMI < 23.6 kg/m^2 (median) and serum LDL-C/HDL-C ratio < 2.08 (75 percentile); group B, BMI ≥23.6 kg/m^2 and serum LDL-C/HDL-C ratio < 2.08; group C, BMI <23.6 kg/m^2 and serum LDL-C/HDL-C ratio ≥ 2.08; group D, BMI ≥ 23.6 kg/m^2 and serum LDL-C/HDL-C ratio ≥ 2.08. We controlled for dependencies between repeated observations in the same subject (MIXED procedure) and used the model with the best fit to our data (Toeplitz covariance structure). We included age as a covariate in the model and we did post hoc tests for multiple pair wise comparisons by Dunnett's method. There were no missing observations in the selected interval (days −10 to + 9) among women with aligned cycles. Differences in means of serum hormones in subgroups of women defined by both BMI and serum LDL-C/HDL-C ratio (groups A-D) were tested for statistical significance in multiple linear regression models and post hoc tests for multiple pair wise comparisons were done by Bonferroni's method.

We used ordinal logistic regression models to estimate the age-adjusted odds ratio (OR), with 95% confidence interval (95% CI), of being in one higher category of breast density associated with an increase of 1 SD in measures of metabolic profile and indices of ovarian hormones. To examine whether each of the statistically significant predictors were independent of known or potential determinants of parenchymal patterns, we used multivariate ordinal logistic regression models.

We evaluated possible interactions between each measure of metabolic profile (measures of adiposity, serum lipids, and serum hormones) and categories (median split for all continuous variables) of age at menarche, age at entry, BMI, truncal fat percentage, energy intake, fat intake, total physical activity, parity (nullipara, 1+ children), current use of alcohol (yes, no) and tobacco (yes, no), and ever use of hormonal contraceptives (yes, no) by including multiplicative interaction terms between the explanatory variables and the category variables in the linear and logistic regression models. Plots of residuals were used to confirm that data fitted to the regression models. We considered results statistically significant when the two-sided $P < 0.05$.

Results

The 206 women included in our study were on average 30.7 years old and had mean age at menarche of 13.1 years (Table 1). Mean BMI was 24.4 kg/m^2. Average length of menstrual cycle in the study was 28.3 days (range, 20-47 days), mean overall average salivary estradiol concentration was 18.2 pmol/L (5.0 pg/mL), and mean overall average salivary progesterone concentration was 131.1 pmol/L (41.2 pg/mL).

Table 1. Means (SD) and proportions of selected characteristics in the study population

Age, y	30.7 (3.1)
Education, total years	16.1 (3.0)
Ethnic minority, Sami, %	7.8
Clinical measures	
Height, cm	167.0 (6.5)
Weight, kg	68.1 (11.7)
BMI, kg/m^2	24.4 (3.8)
Systolic blood pressure, mm Hg	113 (11)
Diastolic blood pressure, mm Hg	71 (8)
Heart rate, per min	69 (11)
Fasting serum glucose, mmol/L	5.0 (0.6)
Menstrual and reproductive characteristics	
Age at menarche, y	13.1 (1.4)
Ever had a full-term pregnancy, %	48.5
Age at first full-term pregnancy, y	24.5 (3.8)
Cycle length, d	28.3 (3.4)
Salivary hormone concentration, pmol/L	
Overall average estradiol	18.2 (9.4)
Average midluteal estradiol	19.9 (11.1)
Overall average progesterone	131.1 (68.7)
Average midluteal progesterone	163.4 (87.7)
Dietary intake	
Energy, kJ/d	8,097 (1,891)
Fat, g/d	77.2 (23.7)
Physical activity, MET-hours/wk	
Occupational physical activity	104 (79)
Household activities	80 (77)
Recreational physical activity	52 (36)
Alcohol consumption	
Teetotaller, %	17.0
Average use among alcohol consumers, units/wk	3.5 (3.4)
Smoking	
Ever smokers, %	45.6
Current smokers, %	22.3
Daily number of cigarettes among smokers	10.1 (5.1)
Previous use of hormonal contraceptives	
Ever users, %	80.6
Total duration of use among ever users, y	5.0 (3.5)

NOTE: The Norwegian EBBA study (n = 206). Number may vary due to missing information.
The conversion factor from mmol/L to mg/dL is 18 for serum glucose. The conversion factor from pmol/L to pg/mL is 0.272 for estradiol and 0.314 for progesterone.

Table 2. Estimated changes in salivary estradiol and progesterone concentrations (pmol/L) with 95% CI by 1 SD increase in explanatory variables

Explanatory variables*	Mean ± SD	Overall average estradiol (n = 206)†	Average midluteal estradiol (n = 192)†,‡	Average midluteal progesterone (n = 192)†,‡
Measures of adiposity				
BMI, kg/m^2	24.4 ± 3.8	2.2 (0.9, 3.5)	2.2 (0.6, 3.8)	−7.1 (−19.5, 5.4)
Truncal fat percentage	32.5 ± 7.6	1.7 (0.4, 3.0)	1.6 (0.1, 3.2)	−8.2 (−20.5, 4.1)
Fasting serum lipids				
Triglycerides, mmol/L	0.86 ± 1.04	1.2 (−0.1, 2.5)	1.4 (−0.2, 2.9)	5.3 (−6.8, 17.5)
Total cholesterol, mmol/L	4.43 ± 0.79	0.8 (−0.6, 2.1)	1.1 (−0.6, 2.7)	4.0 (−8.8, 16.8)
LDL-C, mmol/L	2.52 ± 0.71	1.2 (−0.1, 2.6)	1.6 (−0.03, 3.2)	1.7 (−11.0, 14.4)
HDL-C, mmol/L	1.54 ± 0.33	−1.5 (−2.8, −0.2)	−1.6 (−3.2, −0.1)	9.1 (−3.3, 21.5)
Total/HDL-C ratio	3.00 ± 0.81	2.1 (0.8, 3.4)	2.3 (0.7, 3.9)	−5.6 (−18.2, 7.1)
LDL-C/HDL-C ratio	1.74 ± 0.70	2.0 (0.7, 3.3)	2.2 (0.7, 3.8)	−4.2 (−16.8, 8.5)
Fasting serum hormones				
Insulin, pmol/L	85 ± 59	1.1 (−0.2, 2.4)	1.0 (−0.6, 2.5)	−3.1 (−15.5, 9.2)
IGF-I, nmol/L	25.0 ± 6.3	−0.6 (−2.0, 0.7)	−0.3 (−1.9, 1.4)	−9.7 (−22.4, 3.1)
Leptin, pmol/L	856 ± 560	1.2 (−0.1, 2.5)	1.3 (−0.3, 2.9)	−7.1 (−19.5, 5.4)
Testosterone, nmol/L	1.5 ± 0.5	0.1 (−1.2, 1.4)	0.8 (−0.7, 2.3)	2.3 (−9.6, 14.2)
DHEA-SO$_4$, nmol/L	4.6 ± 2.1	0.7 (−0.6, 2.1)	1.7 (0.1, 3.3)	9.3 (−3.3, 22.0)

NOTE: The Norwegian EBBA study. Age-adjusted linear regression analysis.
The conversion factor from pmol/L to pg/mL is 0.272 for estradiol and 0.314 for progesterone. The conversion factor from mmol/L to mg/dL is 88.6 for triglycerides and 38.7 for total cholesterol and HDL-C. The conversion factor from pmol/L to milliunits/L is 0.17 for insulin. The conversion factor from nmol/L to ng/dL is 765 for IGF-I. The conversion factor from pmol/L to ng/dL is 1.5 for leptin. The conversion factor from nmol/L to ng/dL is 28.8 for testosterone and 36.8 for DHEA-SO$_4$.
Abbreviation: DHEA-SO$_4$, dehydroepiandrostendione sulfate.
*Age at entry and measurements at day 1 to day 5 after onset of the menstrual cycle.
†Numbers may vary due to missing serum values.
‡Includes women with aligned cycles only. Aligned cycle days +5 to +9.

In correlation analyses (not presented in tables), serum HDL-C was inversely related to measures of adiposity (P < 0.001), serum insulin (P < 0.01), leptin (P < 0.001), and DHEA-SO$_4$ (P < 0.05), whereas serum LDL-C was directly related to most of these variables (P < 0.001 for measures of adiposity; P < 0.05 for leptin and DHEA-SO$_4$). Both BMI and truncal fat percentage were directly related to serum insulin and leptin (P < 0.001), and BMI was also directly related to serum DHEA-SO$_4$ level (P < 0.05).

In age-adjusted linear regression analyses, HDL-C was the only serum lipid that was associated with salivary estradiol concentration (Table 2). By each 0.33 mmol/L (12.8 mg/dl; 1 SD) increase in serum HDL-C overall average estradiol concentration dropped by 1.5 pmol/L (0.4 pg/mL; P = 0.02), which equals an 8.2% change in mean overall average estradiol concentration in the study population, and there was a similar drop in average midluteal estradiol concentration (P = 0.04). We observed even stronger relationships between the ratio of serum total/HDL-C and LDL/HDL-C and salivary estradiol concentration (P < 0.01, for overall average and midluteal estradiol for both ratios). Both BMI and truncal fat percentage were positively associated with overall average and midluteal salivary estradiol concentration. BMI was the strongest predictor of overall average estradiol; by each 3.8 kg/m^2 (1 SD) increase in BMI the overall average estradiol concentration increased by 2.2 pmol/L (0.6 pg/mL), which equals a 12.1% change in mean overall average estradiol concentration in the study population (P < 0.001). Moreover, serum DHEA-SO$_4$ was a predictor of average midluteal salivary estradiol concentration (P = 0.04), whereas serum insulin and leptin tended to predict overall average salivary estradiol concentration (P = 0.11 and P = 0.06, respectively). Serum variables and measures of adiposity were unrelated to salivary progesterone levels. When we added age at menarche, parity, energy intake, total physical activity, average use of alcohol, current smoking status (yes/no), and accumulated number of years on hormonal contraceptives to the linear regression models, the associations remained statistically significant with only small modifications of the estimates (not presented in tables).

In age-adjusted analysis of overall average salivary estradiol concentration, there was an interaction between BMI (median split: <23.6 kg/m^2, ≥23.6 kg/m^2) and serum HDL-C ($P_{interaction}$ = 0.03; Table 3). Among women with BMI above 23.6 kg/m^2, overall average estradiol concentration dropped by 2.4 pmol/L (0.7 pg/mL; 13.2% change in mean for the total population P = 0.03) by each 0.33 mmol/L (12.8 mg/dl; 1 SD) increase in serum HDL-C. Serum total/HDL-C ratio and LDL-C/HDL-C ratio were positively associated with overall average estradiol concentration among the heaviest women (P = 0.02 for both), but tests of interaction were not statistically significant. In analysis adjusted for BMI (continuous term), the association between serum LDL-C/HDL-C ratio and overall average estradiol concentration in women with BMI above 23.6 kg/m^2 remained (P = 0.04). There was an interaction between the dichotomised BMI variable and quartiles of

Table 3. Estimated changes in salivary overall average estradiol concentration (pmol/L) with 95% CI by 1 SD increase in serum lipids according to BMI category

Serum lipids	Mean ± SD	BMI < 23.6 (kg/m^2)	BMI ≥ 23.6 (kg/m^2)		P for interaction
LDL-C, mmol/l	2.52 ± 0.71	−0.3 (−1.9, 1.3)*	1.7 (−0.5, 3.9)*	1.5 (−0.6, 3.7)†	0.13
HDL-C, mmol/l	1.54 ± 0.33	0.6 (−1.0, 2.2)*	−2.4 (−4.5,−0.3)*	−2.0 (−4.2, 0.3)†	0.03
Total/HDL-cholesterol ratio	3.00 ± 0.81	−0.3 (−2.4, 1.8)*	2.3 (0.4, 4.3)*	2.0 (−0.1, 4.0)†	0.08
LDL/HDL-cholesterol ratio	1.74 ± 0.70	−0.5 (−2.5, 1.5)*	2.4 (0.4, 4.4)*	2.1 (0.1, 4.1)†	0.05

NOTE: The Norwegian EBBA study (n = 203). Age-adjusted linear regression analysis.
The conversion factor from pmol/L to pg/mL is 0.272 for estradiol. The conversion factor from mmol/L to mg/dL is 38.7 for HDL-C.
*Adjusted for age.
†Adjusted for age, BMI (continuous scale).

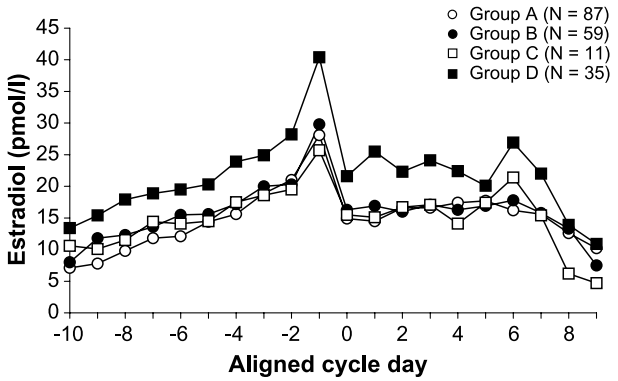

Figure 1. The Norwegian EBBA study (192 aligned cycles). Average salivary estradiol concentration by cycle day in cycles of women categorized by BMI and serum LDL-C/HDL-C ratio. $P = 0.001$ for difference in average estradiol concentration by cycle day between cycles of women characterized by BMI $\geq$ 23.6 kg/m^2 and LDL-C/HDL-C ratio $\geq$ 2.08 (group D) and cycles of women in the other categories (groups A-C). Confidence intervals were omitted for clarity. The conversion factor from pmol/L to pg/mL is 0.272 for estradiol. ○, Group A: BMI < 23.6 kg/m^2 and LDL-C/HDL-C ratio < 2.08; ●, Group B: BMI $\geq$ 23.6 kg/m^2 and LDL-C/HDL-C ratio < 2.08; □, Group C: BMI < 23.6 kg/m^2 and LDL-C/HDL-C ratio $\geq$ 2.08; ■, Group D: BMI $\geq$ 23.6 kg/m^2 and LDL-C/HDL-C ratio $\geq$ 2.08.

serum total/HDL-C ratio ($P_{interaction} = 0.02$) and quartiles of serum LDL-C/HDL-C ratio ($P_{interaction} = 0.007$).

Women characterized by both relatively high BMI (≥23.6 kg/m^2; median split) and high serum LDL-C/HDL-C ratio (≥2.08; 75 percentile) had markedly higher average salivary estradiol levels by cycle day than the rest of the study population from a graphical illustration (Fig. 1) and by generalized linear regression analysis ($P = 0.001$, age adjusted). We examined how the average estradiol levels by cycle day in women with both high BMI and high serum LDL-C/HDL-C ratio (group D) differed from the levels in each of the other groups of women described in Fig. 1 and observed significant differences for all comparisons (group A, $P = 0.002$; group B, $P = 0.02$; group C, $P = 0.03$). Among women with high serum LDL-C/HDL-C ratio (groups C and D), there was no significant difference in mean serum LDL-C/HDL-C ratio by BMI category (median split, 23.6 kg/m^2; $P = 0.07$).

Women characterized by having the highest salivary estradiol levels (group D) had higher levels of serum insulin, leptin and DHEA-SO_4 compared with the rest of the study population as a whole (groups A-C, Fig. 2). However, mean serum insulin and leptin did not differ statistically significantly between the two groups of women with BMI above median (groups B and D). Furthermore, mean serum DHEA-SO_4 did not differ statistically significantly between the two groups of women with serum LDL-C/HDL-C ratio in the highest quartile (groups C and D). The remaining comparisons of mean serum insulin, leptin, and DHEA-SO_4 levels among group D women versus women in the other groups were statistically significant (results not shown).

In multiple logistic regression analyses adjusted for age, age at menarche, parity (nulliparous, parous), and ever use of hormonal contraceptives (yes, no; Table 4), a 3.8 kg/m^2 (1 SD) increase in BMI was associated with a decrease of 64% (OR, 0.36; 95% CI, 0.26-0.49) in the odds of having breasts with density in one higher category, a 0.33 mmol/L (12.8 mg/dl; 1 SD) increase in serum HDL-C was associated with an increase of 63% (OR, 1.63; 95% CI, 1.24-2.14) in the odds of having breasts with density in one higher category, and a 87.6 pmol/L (27.5 pg/mL; 1 SD) increase in average midluteal salivary progesterone concentration was associated with an increase of 36% (OR, 1.36; 95% CI, 1.03-1.81) in the odds of having breasts with density in one higher category. We did not observe any associations between salivary estradiol levels and mammographic breast density.

Discussion

To our knowledge, this is the first report showing that a metabolic profile with low serum HDL-C is related to increased levels of free, biologically active estradiol throughout an entire menstrual cycle. Moreover, we observed that women with both a relatively high BMI (≥23.6 kg/m^2; median split) and a relatively high serum LDL/HDL-C ratio

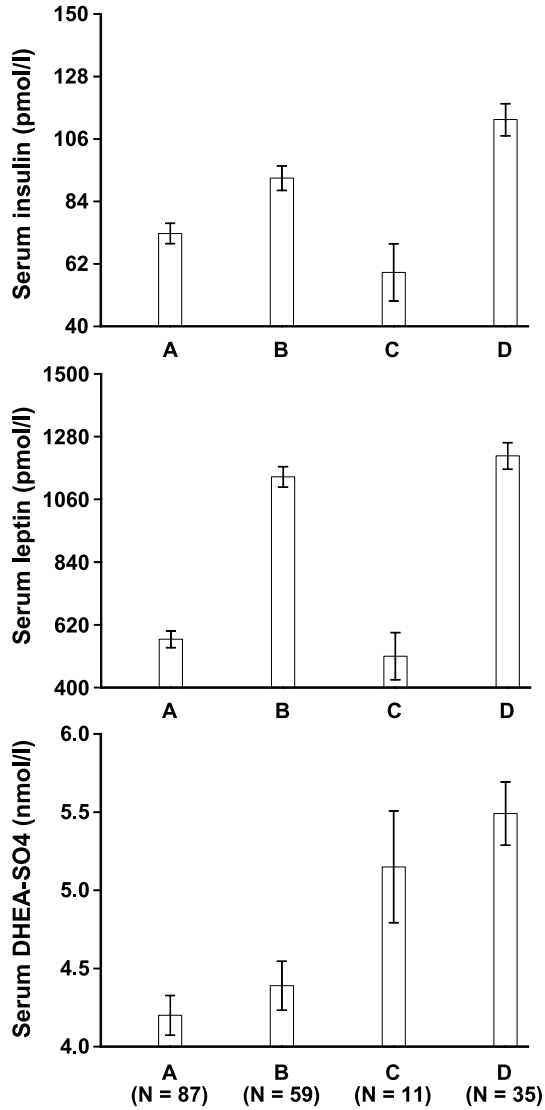

Figure 2. Women with aligned cycles, the Norwegian EBBA study (n = 192). Age-adjusted values of mean serum insulin, leptin, and DHEA-SO_4 (±SE) in women categorized by BMI and serum LDL-C/HDL-C ratio. The conversion factor from pmol/L to milliunits/L is 0.17 for insulin. The conversion factor from pmol/L to ng/dL is 1.5 for leptin. The conversion factor from nmol/L to ng/mL is 36.8 for DHEA-SO_4. Group A: BMI < 23.6 kg/m^2 and LDL-C/HDL-C ratio < 2.08; Group B: BMI $\geq$ 23.6 kg/m^2 and LDL-C/HDL-C ratio < 2.08; Group C: BMI < 23.6 kg/m^2 and LDL-C/HDL-C ratio $\geq$ 2.08; Group D: BMI $\geq$ 23.6 kg/m^2 and LDL-C/HDL-C ratio $\geq$ 2.08.

Table 4. Estimated ORs (95% CI) for being in one higher category of breast density (Modified Wolfe's classification) by 1 SD increase in explanatory variables estimated by sequential logistic regression analysis

	Mean ± SD	OR* 95% CI	OR† 95% CI
Measures of adiposity			
BMI, kg/m^2	24.4 ± 3.8	0.34 (0.25, 0.46)	0.36 (0.26, 0.49)
Truncal fat percentage	32.6 ± 7.6	0.35 (0.26, 0.47)	0.38 (0.28, 0.51)
Fasting serum variables			
HDL-cholesterol, mmol/L	1.53 ± 0.33	1.77 (1.36, 2.32)	1.63 (1.24, 2.14)
Total/HDL-C ratio	3.01 ± 0.80	0.54 (0.41, 0.70)	0.57 (0.43, 0.74)
LDL-C/HDL-C ratio	1.75 ± 0.70	0.60 (0.46, 0.78)	0.64 (0.49, 0.83)
Insulin, pmol/L	85 ± 59	0.66 (0.50, 0.87)	0.68 (0.52, 0.89)
Leptin, pmol/L	858 ± 561	0.49 (0.37, 0.64)	0.52 (0.39, 0.69)
Salivary hormones, pmol/L			
Overall estradiol	18.3 ± 9.4	1.00 (0.78, 1.28)	1.09 (0.84, 1.41)
Midluteal estradiol	20.0 ± 11.1	0.89 (0.68, 1.15)	0.97 (0.74, 1.27)
Overall progesterone	131.4 ± 68.7	1.33 (1.02, 1.73)	1.32 (1.01, 1.73)
Midluteal progesterone	164.0 ± 87.6	1.41 (1.07, 1.86)	1.36 (1.03, 1.81)

NOTE: The Norwegian EBBA study (n = 205). ORs shown are for each measure of adiposity, serum variable, and salivary hormone after adjustments.
The conversion factor from mmol/L to mg/dL is 38.7 for HDL-C. The conversion factor from pmol/L to milliunits/L is 0.17 for insulin. The conversion factor from pmol/L to ng/dL is 1.5 for leptin. The conversion factor from pmol/L to pg/mL is 0.272 for estradiol and 0.314 for progesterone.
*Adjusted for age.
†Adjusted for age, age at menarche, parity (nullipara, parous), and past use of hormonal contraceptives (ever, never).

(≥2.08; 75 percentile) were exposed to far higher levels of free estradiol than other women. In a large prospective study, we recently documented an increased risk of postmenopausal breast cancer by decreasing serum HDL-C among overweight and obese women (2); previous results from prospective studies are limited and not stratified by BMI categories (15-17). However, several studies have reported lower levels of serum HDL-C in breast cancer patients versus controls (18-20).

Thus, by analyzing the associations between metabolic profile and levels of free estradiol in the present cross-sectional data we had the possibility to identify hypothesized physiologic mechanisms (2) that may link overweight/obesity and dyslipidemia to breast cancer risk in women. As excessive exposure to estrogens is a major stimulus of breast carcinogensis, we suggest that low serum HDL-C is a true biomarker of breast cancer risk that may be most useful among overweight and obese women.

Circulating estrogen and progesterone are thought to play a major role in breast carcinogenesis as the risk of breast cancer increases with early menarche and late menopause (6). Bilateral oophorectomy before the age of 35 reduces the lifetime risk of breast cancer in a woman by nearly 75% (21). A reanalysis of nine prospective studies observed a 2.6 times increase in the risk of postmenopausal breast cancer among women with free serum estradiol in the highest quintile as compared with women with free serum estradiol in the lowest quintile (22). Results from studies of premenopausal breast cancer are less conclusive and are complicated by the cyclic hormonal variation occurring during the menstrual cycle. An increased risk of breast cancer in premenopausal women with relatively high levels of estradiol has been suggested (23-25). The mechanisms by which estrogens cause breast cancer have not been firmly established but the prevailing theory proposes that estrogens increase the rate of cell proliferation by stimulating estrogen receptor-mediated transcription and thereby the number of errors occurring during DNA replication (26, 27).

The sample sizes of previous studies that have examined estradiol levels in blood have been small. Thomson et al. (28; n = 24; 21 samples during one cycle), Shelley et al. (29; n = 363; one sample in early follicular phase), and Semmens et al. (30; n = 36; one sample) did not observe any association between serum HDL-C and estradiol. In contrast, Gorbach et al. (31; n = 24; one sample in follicular phase) and Lyons Wall et al. (32; n = 12; 20 samples during one cycle) observed a positive association between serum HDL-C and estradiol.

Interestingly, there was a linear increase in salivary estradiol concentration related to increasing BMI and truncal fat percentage in our study.[7] Studies have generally found relatively stable and uniform estradiol levels among normal and overweight women, whereas obesity has been associated with impaired ovarian function and a decrease in serum estradiol (33-35). However, Kirschner et al. (36) and Leenen et al. (37) found that free estradiol levels in women with abdominal obesity were higher than in subjects with lower-body (gluteal) obesity.

One explanation for the difference in findings between studies of both serum HDL-C and BMI and levels of estradiol may be the use of different estimates of endogenous estradiol exposure; as serum estradiol includes both the protein bound fraction and the free fraction of the hormone, salivary estradiol is probably a better predictor of free, biologically active estradiol level (38). Furthermore, the association between estradiol and serum HDL-C may be dependent on metabolic profile (i.e., overweight/obesity) and the presence of other hormones (i.e., insulin); thus, stratified analysis may be most appropriate. Finally, our work is based on relatively young birth cohorts who in comparison to past generations have a more sedentary lifestyle that favors metabolic disturbances (39). Numerous studies have reported various patterns of cyclic fluctuations in serum HDL-C as a function of the phase of the menstrual cycle (32, 40); however, Reed et al. (41) found that the intraindividual variability for serum HDL-C in premenopausal women was similar to that found in men and postmenopausal women and differences between studies in timing of serum HDL-C assessment according to cycle phase are not likely to have influenced our results.

Our study shows that serum HDL-C is inversely related to level of serum DHEA-SO_4 and is in agreement with others (28, 30). The observed associations between serum HDL-C and salivary estradiol and serum DHEA-SO_4 are biologically plausible as sex steroids are physiologic regulators of serum lipids (42). The changes in serum HDL-C levels induced by sex steroids may be mediated, in part, by the lipolytic enzyme, hepatic lipase; the activity of this enzyme is regulated by sex steroids (43). The study of sex differences in cardiovascular disease has revealed that androgens are the key modulators of serum lipid levels and in particular, of serum HDL-C levels (44). Women with relative androgen excess (i.e., polycystic ovary syndrome) have lower levels of serum HDL-C than women with normal ovarian function (44). In the present study, serum DHEA-SO_4 was directly related to average

midluteal estradiol concentration; this supports the hypothesis (2) that the conversion of androgens to estrogens in adipose tissue might be an underlying mechanism for the increased risk of breast cancer by low HDL-C. The present study also suggests that other hormonal changes, particularly hyperinsulinemia, in the heaviest women (BMI $\geq$ 23.6 kg/m^2) may be of importance; insulin enhances ovarian function and lowers the level of SHBG (45).

In this cross-sectional study, serum LDL-C/HDL-C ratio was the index of HDL-C level that was the strongest predictor of salivary estradiol concentration and this ratio may be a more sensitive marker of breast cancer risk than serum HDL-C alone. Higher levels of serum LDL-C among breast cancer patients have been observed in retrospective studies (19, 46), but not in prospective studies (15, 16). Serum LDL-C/HDL-C ratio may reflect aspects of the serum lipid profile that are especially strongly influenced by sex steroid levels.

The observed increase in levels of estradiol associated with potential markers of increased breast cancer risk (high BMI, low HDL-C, and high LDL-C/HDL-C ratio) was not accompanied by an increase in progesterone levels. This suggests that the markers may also be relevant for endometrial cancer as the balance of estrogens and progesterone largely influences endometrial cancer risk (10).

Our finding of a direct relationship between salivary progesterone levels and breast density is in accordance with studies of serum progesterone (47). This supports the role of progesterone in breast carcinogenesis, as mammographic density is an independent predictor of breast cancer risk, with increase in risk by increasing density (48, 49). The fact that estrogen plus progestin use increases both mammographic breast density and the incidence rate of breast cancer versus estrogen alone (50) or placebo (50, 51) underscores the biological significance of our result. Salivary estradiol concentration was not associated with breast density in our study as also reported by others (47).

In our study, breast density was negatively associated with measures of adiposity and positively associated with serum HDL-C, and, importantly, the associations were not influenced by parity. The impact of fat mass on breast density is well known from several studies in both premenopausal and postmenopausal women (52-54). A direct relationship between serum HDL-C and mammographic dysplasia was also observed by Boyd et al. (55) independently of percentage body fat. Thus, studies indicate that potential markers of breast cancer risk (high BMI, low serum HDL-C, and high serum LDL-C/HDL-C ratio) are negatively associated with high-risk mammographic parenchymal patterns. This negative confounding of potential markers of increased breast cancer risk may mean that the effect of parenchymal patterns on risk will tend to be underestimated unless adjusted for metabolic profile (BMI and serum HDL-C) and vice versa, as suggested by Sala et al. (54).

Our study is strengthened by the estimation of daily estradiol and progesterone concentrations in saliva using well-developed and validated methods and assays to characterize the women's exposure to free, biologically active ovarian steroids, and the comparison of levels by aligned cycle days in the large majority of the population (13, 14). Furthermore, salivary levels of estradiol and progesterone are quite stable within subjects over time (38). We registered all clinical variables, including mammogram, within the same narrow frame of the cycle in each participant to minimize any influence of cycle phases on parenchymal breast density, in particular (56). To limit potential influence of season, women did not participate during months with little daylight (December and January). Associations between BMI and serum lipids were comparable with our observations in a large prospective study of metabolic risk profiles among women of the same age and from the same geographic area (57). In our study, misclassification of mammographic density is likely to have been minimal, due to the stable work by one expert reader and as we also observed strong associations between mammographic parenchymal pattern and established risk factors for breast cancer (results not shown).

In our recent prospective study (2), from which the study hypothesis originated, the increased risk of postmenopausal breast cancer associated with low serum HDL-C was found among overweight and obese women (BMI $\geq$25 kg/m^2). In this cross-sectional study, we used another BMI cutoff ($\geq$23.6 kg/m^2) to define the heaviest women; the women were on average 13 years younger than in the follow-up study (2) and there is a physiologic increase in BMI by increasing age at least up to age 60 years in females (1, 58). Thus, it is reasonable that associations observed for younger women in lower BMI categories may be translated to older women in higher BMI categories.

In conclusion, our results support the hypothesis based on our recently published results from a large prospective study on breast cancer (2), that low serum HDL-C in overweight and obese women is associated with higher levels of breast mitogens and estrogens, in particular, and therefore is a biologically sound marker of breast cancer risk that may be used to identify high risk individuals that may be candidates for intervention.

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