Research Article

Cancer Prevention Research

High-Density Lipoprotein-Cholesterol, Daily Estradiol and Progesterone, and Mammographic Density Phenotypes in Premenopausal Women

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Abstract

High-density lipoprotein-cholesterol (HDL-C) may influence the proliferation of breast tumor cells, but it is unclear whether low HDL-C levels, alone or in combination with cyclic estrogen and progesterone, are associated with mammographic density, a strong predictor of breast cancer development. Fasting morning serum concentrations of HDL-C were assessed in 202 premenopausal women, 25 to 35 years of age, participating in the Norwegian Energy Balance and Breast Cancer Aspects (EBBA) I study. Estrogen and progesterone were measured both in serum, and daily in saliva, throughout an entire menstrual cycle. Absolute and percent mammographic density was assessed by a computer-assisted method (Madena), from digitized mammograms (days 7–12). Multivariable models were used to study the associations between HDL-C, estrogen and progesterone, and mammographic density phenotypes. We observed a positive association between HDL-C and percent mammographic density after adjustments ($P = 0.030$). When combining HDL-C, estradiol, and progesterone, we observed among women with low HDL-C (<1.39 mmol/L), a linear association between salivary 17β-estradiol, progesterone, and percent and absolute mammographic density. Furthermore, in women with low HDL-C, each one SD increase of salivary mid-menstrual 17β-estradiol was associated with an OR of 4.12 (95% confidence intervals; CI, 1.30–13.0) of having above-median percent (28.5%), and an OR of 2.5 (95% CI, 1.13–5.50) of having above-median absolute mammographic density (32.4 cm^2). On the basis of plausible biologic mechanisms linking HDL-C to breast cancer development, our findings suggest a role of HDL-C, alone or in combination with estrogen, in breast cancer development. However, our small hypothesis generating study requires confirmation in larger studies. *Cancer Prev Res; 8(6); 535–44. ©2015 AACR.*

Introduction

Breast cancer development has been linked to high-density lipoprotein-cholesterol (HDL-C; ref. 1), although the findings are somewhat contradictory (2, 3). Low levels of HDL-C, which transport and store cholesterol (4), have been associated with low-grade inflammation and proinflammatory cytokines (5–7), which may stimulate breast cell proliferation. High levels of the cholesterol metabolite 27-hydroxycholesterol were observed to increase estrogen-dependent breast cancer proliferation (8, 9). Interestingly, mammographic density, a strong predictor of breast cancer development, is positively correlated with the number of epithelial cells (10), and mammographic density was recently linked to metabolic syndrome (11).

Mammographic density refers to the structure and relationship of the adipose, epithelial, and stromal tissues (12, 13). Percent mammographic density reflects relative amounts of fibroglandular and fat tissue, and absolute mammographic density reflects epithelial and stromal tissues, the dense areas of the breast (14, 15). Importantly, there is a clear tendency for ductal carcinoma *in situ*, and invasive breast cancer to occur in areas that are mammographically dense (16). Of note, absolute mammographic density, as compared with percent mammographic density, may be less confounded by body fat (17, 18). However, it is unclear whether absolute mammographic density, compared with percent mammographic density, is a more suitable marker of breast cancer development, when studying factors such as variations in HDL-C levels, associated with metabolic syndrome (15, 19, 20).

Estrogen and progesterone have been observed to induce the proliferation of breast epithelial cells (12), to be associated with HDL-C (21), and with mammographic density (22–24). Recently, estrogen and mammographic density were observed, independently, to be associated with breast cancer development (25). However, it is less known whether HDL-C, is associated with mammographic density, in particular for premenopausal women (26, 27). We have previously studied the association between cyclic estrogen and an unfavorable metabolic profile (21, 28), and

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observed that HDL-C was inversely associated with cyclic estrogen (21). The complexity of assessing cyclic hormones throughout an entire menstrual cycle among premenopausal women underlines the importance of inclusion of both total serum levels (bound) and direct measurements of unbound levels of salivary hormones.

On the basis of recent observations (11, 25, 29) and biologic mechanisms hypothesized (1, 7, 30), the main aim of this exploratory hypothesis generating study was to explore whether differences in HDL-C, alone or in combination with cyclic estrogen and progesterone, assessed both in serum and in saliva, were associated with mammographic density phenotypes among premenopausal women.

Materials and Methods

Participants and study design

The participating women in the Norwegian Energy Balance and Breast Cancer Aspect (EBBA)-I Study (2000–2002), were recruited through local media campaigns (21). A total of 204 women ages 25 to 35 years who met the following criteria: regular menstrual cycles (22–38 days within the previous 3 months), no use of any regular (daily/weekly) medication, no pregnancy, lactation, or use of steroid contraceptives over the previous 6 months, and no history of gynecologic or chronic disorders (e.g., diabetes, hypo/hyperthyroidism, polycystic ovary syndrome) were included, (21). Two women were excluded, due to missing mammographic data, leaving data from 202 premenopausal women available for the present study. Validated and standardized questionnaires (self- and interviewer- administered by trained personnel) were used to collect information about reproductive history, previous hormone use, diet, and lifestyle habits (21, 28, 31).

Clinical parameters

The participants were clinically examined on the first possible day after onset of menstrual bleeding, by one trained nurse and the same two physicians (A.-S. Furberg andI. Thune) at the Clinical Research Center, University Hospital of North Norway (UNN), Tromsø, Norway. The participants underwent clinical examinations at three scheduled visits over the course of one menstrual cycle: first visit (days 1–5 of the menstrual cycle, early follicular phase), second visit (days 7–12, late follicular phase), and third visit (days 21–25, late luteal phase). Overnight fasting blood samples were collected and analyzed (21). 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. Blood pressure was measured (PROPAQ 104) with participants sitting in a resting position. 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).

Assessment of serum HDL-C, total cholesterol, and triglycerides

Lipids were measured in fresh serum using kits from Roche Diagnostics GmbH. HDL-C was quantified by direct assay, using enzymes modified by polyethylene glycol and dextran sulfate. The coefficient of variation (CV) for HDL-C measurement was approximately 3%. Total cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Serum triglycerides were assayed by enzymatic hydrolysis with lipase.

Assessment of estrogen and progesterone

Fasting morning serum concentrations of female sex steroid hormones (17β-estradiol, progesterone) were measured at the three scheduled visits during the menstrual cycle. Serum concentrations of 17β-estradiol and progesterone were measured using a direct immunometric assay (Immuno-1), from Bayer Diagnostics (21). The sensitivity for estradiol was 0.01 nmol/L and the CV was 3.9%. The sensitivity and CV for progesterone were 0.13 nmol/L and 5.7%, respectively. Sex hormone-binding globulin (SHBG) was measured by an immunometric method (both Diagnostic Products Corporation-Bierman GmbH) with a CV of 5% to 10%.

The participants collected daily morning saliva samples over one menstrual cycle, starting the first day of menstrual bleeding, using validated protocols developed at the Reproductive Ecology Laboratory at Harvard University (Cambridge, MA; refs. 21, 32). The samples were stored at −70°C. All samples were run in duplicate, and samples from the same cycles were run within the same assay. The assays were done in different batches. 17β-estradiol and progesterone concentrations were measured in daily saliva samples using a ^{125}I-based radioimmunoassay kit (#39100, Diagnostic Systems Laboratory).

All cycles were aligned to the day of ovulation, based on the identification of the drop in 17β-estradiol. This provides a reasonable estimate of the day of ovulation for women with both short and long menstrual cycle lengths (33). This drop in 17β-estradiol, could not be made out for 14 women; hence, their cycles were not aligned. Overall, mean salivary 17β-estradiol concentration was calculated for all 204 women, whereas additional indices (i.e., luteal index, follicular index, AUC, and mid-menstrual 17β-estradiol on days −7 to +6) within the same menstrual cycle were calculated for 188 women with aligned cycles and mammograms.

The sensitivity of the 17β-estradiol assay was 4 pmol/L, and average intra-assay CV was 9%. The measurements of 17β-estradiol had higher CVs at the start and end of the menstrual cycle, and the interassay variability ranged from 23% (low pool) to 13% (high pool). Furthermore, there were higher rates of missing data at the end of the cycle, thus we included aligned 17β-estradiol salivary measurements from day −7 to day +6 in this study. The sensitivity of the salivary progesterone assay was 13 pmol/L, and average intra-assay CV was 10%. Interassay CV ranged from 19% (low pool) to 12% (high pool). Because of higher CVs and missing data at the end of the cycle, we included salivary progesterone measurements from day 0 to day +9.

Assessment of mammographic density

Bilateral two-view mammograms were obtained between cycle days 7 and 12, at the Centre of Breast Imaging, UNN, using a standard protocol (21, 34). The left craniocaudal mammograms were digitized, and imported into a computerized mammographic density assessment program (Madena) University of Southern California School of Medicine (Los Angeles, CA; refs. 14, 15). Density measurements were conducted by a trained reader (G. Ursin). Total breast area was defined using a special outlining tool, and the Madena software estimated the size in cm^2 of this area. In order to assess density, the reader outlined a region of interest (ROI), excluding the pectoralis muscle, prominent veins, and fibrous strands. The reader, blinded to any study characteristics of the population, applied a tinting tool to pixels considered to represent dense areas of the mammograms within the ROI.

Table 1. Characteristics of the study population by tertiles of HDL-C (mmol/L)

Study characteristics	HDL-C <1.39 ($n = 66$)[a]	HDL-C 1.39–1.67 ($n = 68$)[a]	HDL-C >1.67 ($n = 65$)[a]	P[b]
Age, y	31.0 (3.09)	30.3 (3.01)	30.9 (3.11)	0.303
Education, total, y	15.8 (3.17)	16.4 (2.94)	15.8 (3.03)	0.400
Reproductive factors[c]				
Age at menarche, y	12.8 (1.32)	13.3 (1.36)	13.3 (1.40)	0.072
Menstrual cycle length, d	28.5 (3.00)	28.5 (3.56)	27.7 (2.98)	0.296
Number of children, no.	1.17 (1.14)	0.69 (0.90)	0.91 (1.30)	0.052
Clinical parameters				
BMI, kg/m^2[d]	26.1 (4.21)	24.0 (3.52)	22.9 (2.63)	<0.001
Height, cm[d]	166 (5.10)	168 (7.27)	167 (7.05)	0.422
Total tissue fat, % (DXA)[e]	37.7 (7.26)	33.4 (7.03)	30.9 (6.97)	<0.001
Systolic blood pressure, mmHg[d]	116 (12.5)	112 (10.9)	111 (9.8)	0.055
Serum samples[d]				
Total cholesterol, mmol/L	4.24 (0.85)	4.27 (0.73)	4.60 (0.67)	0.021
Cholesterol/HDL-C ratio	3.67 (0.89)	2.93 (0.47)	2.43 (0.43)	<0.001
Triglycerides, mmol/L	0.94 (0.55)	1.00 (1.68)	0.65 (0.23)	0.123
CRP, nmol/L	5.55 (5.01)	4.85 (3.92)	4.68 (2.00)	0.413
SHBG, nmol/L	46.2 (18.3)	50.5 (16.5)	59.4 (21.9)	<0.001
Serum hormones[f]				
Estradiol, early follicular, nmol/L	0.157 (0.080)	0.138 (0.036)	0.146 (0.061)	0.215
Estradiol, late follicular, nmol/L	0.363 (0.273)	0.481 (0.348)	0.486 (0.307)	0.040
Estradiol, luteal, nmol/L	0.404 (0.200)	0.453 (0.199)	0.434 (0.202)	0.369
Progesterone, early follicular, nmol/L	5.97 (7.62)	3.49 (2.93)	5.17 (7.30)	0.068
Progesterone, late follicular, nmol/L	4.54 (6.39)	5.54 (8.51	5.82 (7.95)	0.607
Progesterone, luteal, nmol/L	30.8 (19.7)	38.5 (19.9)	38.6 (20.2)	0.037
Salivary hormones				
Mid-menstrual estradiol, pmol/L[g]	20.3 (10.1)	17.3 (8.81)	17.4 (7.64)	0.107
Luteal progesterone, pmol/L[h]	141 (81.0)	137 (76.0)	154 (61.7)	0.443
Lifestyle factors[c]				
Current smokers, %	30.3	10.6	25.4	0.018
Alcohol units per week, U	1.96 (2.54)	3.06 (3.29)	3.71 (4.09)	0.012
Energy intake, kJ/d	7893 (1,898)	8,066 (1,928)	8,460 (1,799)	0.210
Previous use of OC, %	87.9	77.3	84.8	0.242
Leisure time MET, h per wk	64.2 (147)	50.7 (35.2)	58.4 (41.9)	0.671
Mammographic density[e]				
Absolute density, cm^2	27.1 (19.2)	40.9 (27.2)	36.6 (21.4)	0.002
Percent density, %	20.9 (16.7)	31.9 (17.6)	36.9 (19.2)	<0.001

Abbreviations: E_2, 17β-estradiol; One MET is defined as the energy cost of sitting quietly and is equivalent to a caloric consumption of 1 kcal/kg/hour; OC, oral contraceptives.

[a]Numbers may vary due to missing information.

[b]One-way ANOVA or χ^2 test, significance level $P < 0.05$.

[c]Questionnaires.

[d]Measurements at days 1–5 after onset of menstrual cycle.

[e]Measurements at days 7–12 after onset of menstrual cycle.

[f]Serum hormone samples after onset of menstrual cycle: early follicular phase (days 1–5 after onset), late follicular phase (days 7–12 after onset), luteal phase (21–25 after onset).

[g]Daily salivary estradiol samples, aligned cycle days −7 to +6.

[h]Daily salivary progesterone samples, aligned cycle days 0 to +9.

The Madena software calculated the size of this dense area in cm^2. Absolute mammographic density represented the number of the tinted pixels. Percent mammographic density was the ratio of absolute mammographic density to the total breast area (area of ROI) multiplied by 100. 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 correlation coefficient of 0.97.

Statistical analysis

In this exploratory hypothesis generating study, based on the plausible biologic mechanisms suggested, linking HDL-C to breast cancer development, and to endogenous sex-steroid levels,

Table 2. The association between HDL-C and absolute (cm^2) and percent mammographic density (%) in uni- and multivariable models ($n = 202$)

	β-Coefficient	(95% CI)	P
Absolute mammographic density (cm^2)			
HDL-C, mmol/L[a]	10.3	(0.49–20.2)	0.040
HDL-C, mmol/L[b]	5.20	(−5.15–15.5)	0.323
HDL-C, mmol/L[c]	5.80	(−4.38–16.0)	0.262
Percent mammographic density (%)			
HDL-C, mmol/L[a]	19.0	(11.5–26.6)	<0.001
HDL-C, mmol/L[b]	7.26	(0.59–13.9)	0.033
HDL-C, mmol/L[c]	7.23	(0.72–13.7)	0.030

[a]Univariable linear regression.

[b]Multivariable linear regression, adjusted for age, BMI, and parity.

[c]Multivariable linear regression, adjusted for age, BMI, parity, smoking, and oral contraceptive use.

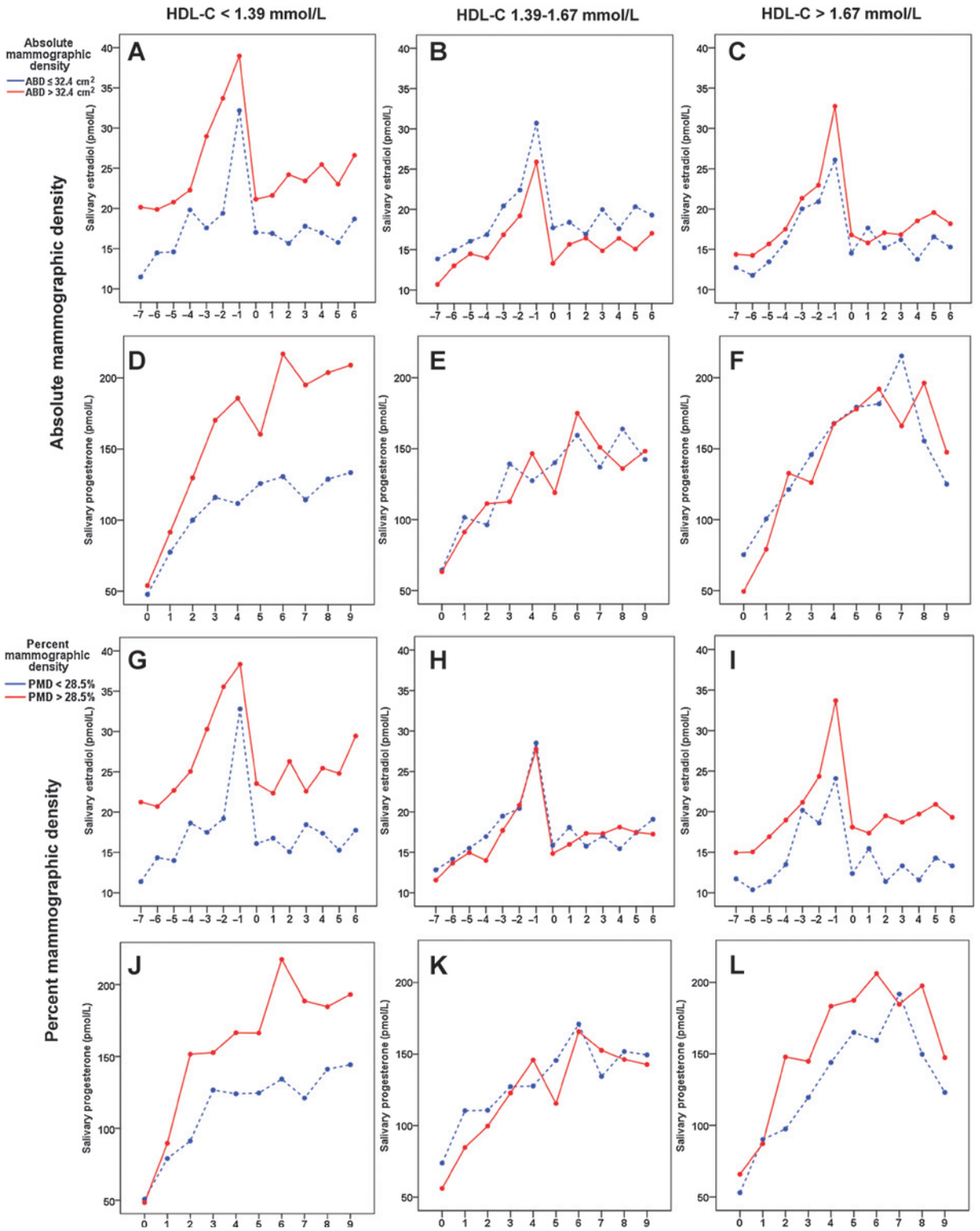

Figure 1.
Daily salivary 17β-estradiol and progesterone throughout an entire menstrual cycle by median split of absolute (≤ or >32.4 cm^2) and median split of percent (≤ or >28.5%) mammographic density stratified by tertiles of HDL-C. 17β-estradiol levels (pmol/L) by absolute mammographic d(AMD). A, HDL-C <1.39 mmol/L ($n = 63$): ≤32.4 cm^2, mean 17.7 pmol/L, >32.4 cm^2, mean 25.0 pmol/L ($P = 0.016$). B, HDL-C 1.39–1.67 mmol/L ($n = 64$): ≤32.4 cm^2, mean 19.0 pmol/L, >32.4 cm^2, mean 15.9 pmol/L ($P = 0.199$). C, HDL-C >1.67 mmol/L ($n = 55$): ≤32.4 cm^2, mean 16.4 pmol/L, >32.4 cm^2, mean 18.7 pmol/L ($P = 0.331$). Progesterone levels (pmol/L) by absolute mammographic density. D, HDL-C <1.39 mmol/L ($n = 63$): ≤32.4 cm^2, mean 109 pmol/L, >32.4 cm^2, mean 162 pmol/L ($P = 0.017$). E, HDL-C 1.39–1.67 mmol/L ($n = 64$): ≤32.4 cm^2, mean 127 pmol/L. >32.4 cm^2, mean 125 pmol/L ($P = 0.923$). (*Continued on the following page.*)

we studied the association between HDL-C, alone and in combination with serum and salivary estrogen and progesterone levels, and the study outcomes; absolute and percent mammographic density, using multivariable linear and logistic regression models. This was done to take into account a potential combined effect of HDL-C and cyclic estrogen and progesterone throughout the menstrual cycle, among premenopausal women in relation to mammographic density phenotypes. Percent mammographic density and absolute mammographic density were used as both continuous and dichotomized variables, representing lower and higher density, using median values as cut-off points: percent mammographic density (28.5%), and absolute mammographic density (32.4 cm^2). Previous studies in premenopausal (35) and postmenopausal (36) women have found a 2- to 3-fold increase in breast cancer risk for women with absolute mammographic density >32 cm^2 (36) and percent mammographic density >25% (35, 36). These observations support the comparison of women with above versus below median absolute and percent mammographic density, as we did in our study. All variables, including mammographic densities and hormone variables, were approximately normally distributed hence no transformations were needed. Moreover, we did not observe any outliers that could drive the associations.

Several models build on previously established observations and recently suggested biologic mechanisms influencing mammographic density phenotypes, were tested (1, 11, 29). These models included a variety of potentially confounding variables such as age (continuous in years), BMI (continuous in kg/m^2), number of children (continuous in number), age at menarche (continuous in years), previous oral contraceptives (OC) use (categorical, yes/no), smoking habits (categorical, yes/no), alcohol intake (continuous U/week), energy intake (continuous kJ/day), and leisure time physical activity [continuous in metabolic equivalents (MET) hours/week).

As low HDL-C (<1.4 mmol/L) has been associated with breast cancer development (1, 37), we studied the associations between HDL-C and mammographic phenotypes by tertiles of HDL-C: HDL-C <1.39 mmol/L, HDL-C 1.39–1.67 mmol/L, and HDL-C >1.67 mmol/. Women within the HDL-C tertiles were compared by characteristics of the study population using one-way ANOVA for continuous variables, and the χ^2 test for categorical variables. Potentially confounding factors were evaluated. Age, BMI, number of children, smoking habits, and OC use were included as covariates in the final multivariable models. Pearson correlation, univariable and multivariable, linear and logistic regression models, in tertiles of HDL-C, were used.

We studied the association between HDL-C, in combination with daily salivary 17β-estradiol and progesterone throughout an entire menstrual cycle, stratified by tertiles of HDL-C and mammographic density, by using linear mixed models for repeated measures. The outcome (absolute and percent mammographic density) was dichotomized (median split) between low and high absolute ($\leq$ or >32.4 cm^2) and low and high percent ($\leq$ or >28.5%) mammographic density. The Toeplitz covariance structure gave the best fit to the data, and was thus used in all models. The AUC for 17β-estradiol and progesterone was calculated for each participant with an aligned cycle using the trapezium rule (38). The present study is based on plausible biologic mechanisms hypothesized and exploratory analysis, resulting in some multiple testing. However, multiple corrections, such as Bonferroni, are in many circumstances considered to be too stringent, and may result in false-negative results (type II errors). Thus, we chose not to adjust for multiple corrections, but we are aware of the risk of false-positive results (type I errors) in this explorative hypothesis generating study. Thus, *P* values were two sided and considered significant if $P < 0.05$. The analyses were conducted with SPSS version 21.0 (IBM Corporation).

Ethics statement

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

Results

The participating premenopausal women had a mean age of 30.6 years, mean serum total cholesterol of 4.45 mmol/L, mean HDL-C of 1.54 mmol/L, mean absolute mammographic density of 34.7 cm^2, and mean percent mammographic density of 29.8%, (results not presented in Table). Selected characteristics of the participating women are presented by tertiles of HDL-C in Table 1. Women in the lowest HDL-C tertile group (<1.39 mmol/L), had a higher BMI, higher systolic blood pressure, and had a lower absolute and percent mammographic density, compared with women in the middle and highest HDL-C tertiles (Table 1). On the basis of the hypothesis that a possible cooccurrence of low HDL-C, proinflammatory factors, and estradiol may exist in the late luteal phase, we examined the association between low HDL-C and inflammatory markers [C-reactive protein (CRP), white blood cells, thrombocytes] and serum/salivary estradiol. However, no associations were observed (results not presented).

We observed a positive association between HDL-C and percent mammographic density after adjustments ($P = 0.030$), whereas the associations between HDL-C and absolute mammographic density disappeared in the multivariable models (Table 2). We found a stronger inverse association between BMI and percent mammographic density (Pearson correlation coefficient, −0.578, $P = <0.001$), than between BMI and absolute mammographic density (Pearson correlation coefficient, −0.230, $P = 0.001$; results not presented in Tables).

We examined the women by tertiles of HDL-C, in combination with mean overall salivary 17β-estradiol and progesterone concentrations, throughout the mid-menstrual phase in relation to absolute and percent mammographic density (Fig. 1A). Women in the lowest HDL-C tertile (<1.39 mmol/L) having above-median absolute mammographic density compared with women in the

(*Continued.*) F, HDL-C >1.67 mmol/L ($n = 55$): $\leq$32.4 cm^2, mean 147 pmol/L. >32.4 cm^2, mean 144 pmol/L ($P = 0.863$). 17β-estradiol levels (pmol/L) by percent mammographic density (PMD). G, HDL-C <1.39 mmol/L ($n = 63$): $\leq$28.5%, mean 17.5 pmol/L. >28.5%, mean 26.3 pmol/L ($P = 0.006$). H, HDL-C 1.39–1.67 mmol/L ($n = 64$): $\leq$28.5%, mean 17.6 pmol/L. >28.5%, mean 17.1 pmol/L ($P = 0.840$). I, HDL-C >1.67 mmol/L ($n = 55$): $\leq$28.5%, mean 14.4 pmol/L, >28.5%, mean 19.9 pmol/L ($P = 0.061$). Progesterone levels (pmol/L) by percent mammographic density. J, HDL-C <1.39 mmol/L ($n = 63$): $\leq$28.5%, mean 114 pmol/L. >28.5%, mean 156 pmol/L ($P = 0.080$). K, HDL-C 1.39–1.67 mmol/L ($n = 64$): $\leq$28.5%, mean 130 pmol/L. >28.5%, mean 123 pmol/L ($P = 0.742$). L, HDL-C >1.67 mmol/L ($n = 55$): $\leq$28.5%, mean 129 pmol/L. >28.5% has mean 155 pmol/L ($P = 0.281$).

Table 3. The associations between salivary and serum estradiol (SD) and progesterone (SD) and absolute mammographic density (cm^2), stratified by tertiles of HDL-C

Variables	Mean (SD)	HDL-C <1.39 ($n = 66$)[a] β-Coefficient (95% CI)	*P*	HDL-C 1.39–1.67 ($n = 68$)[a] β-Coefficient (95% CI)	*P*	HDL-C >1.67 ($n = 65$)[a] β-Coefficient (95% CI)	*P*
Estradiol (E_2)							
Saliva, pmol/L[b]							
Mid-menstrual, days −7 to +6	18.2 (8.98)	3.99 (0.19–7.81)	0.041	−6.01 (−13.0–0.97)	0.090	3.39 (−2.90–9.69)	0.283
Follicular phase, days −7 to −1	19.0 (9.58)	4.67 (0.97–8.36)	0.014	−6.13 (−13.9–1.65)	0.120	4.10 (−2.29–10.5)	0.203
Luteal phase, days 0 to +6	17.4 (9.22)	2.79 (−1.14–6.72)	0.161	−5.49 (−11.9–0.90)	0.091	2.38 (−3.77–8.52)	0.440
$AUC_{through\ cycle}$, time × pmol/L	269 (133)	4.09 (0.30–7.89)	0.035	−6.09 (−13.1–0.92)	0.087	3.69 (−2.61–9.99)	0.244
Serum, nmol/L							
Early follicular[c]	0.15 (0.06)	−0.56 (−3.82–2.71)	0.734	−1.91 (−13.2–9.36)	0.736	2.77 (−1.90–7.44)	0.240
Late follicular[d]	0.44 (0.31)	−1.42 (−6.49–3.65)	0.577	−1.79 (−7.82–4.25)	0.556	−2.03 (−7.04–2.98)	0.421
Late luteal[e]	0.43 (0.20)	2.08 (−2.00–6.17)	0.311	1.34 (−6.32–8.99)	0.728	1.16 (−4.11–6.43)	0.661
Progesterone							
Saliva, pmol/L[b]							
Luteal, days 0 to +9	142 (73.5)	4.31 (0.46–8.16)	0.029	−1.06 (−7.77–5.65)	0.752	0.93 (−5.32–7.17)	0.767
$AUC_{through\ cycle}$, time × pmol/L	1341 (718)	4.36 (0.54–8.18)	0.026	−1.86 (−8.71–4.99)	0.589	−0.00 (−6.14–6.14)	0.999
Serum, nmol/L							
Early follicular[c]	4.83 (6.29)	1.42 (−2.10–4.94)	0.422	−2.73 (−16.9–11.5)	*0.703*	−0.04 (−4.09–4.01)	0.985
Late follicular[d]	5.24 (7.54)	−2.71 (−7.82–18.8)	0.293	5.80 (−0.31–11.9)	*0.062*	3.71 (−0.71–8.13)	0.098
Late luteal[e]	35.6 (20.1)	2.18 (−2.18–6.54)	0.321	−4.57 (−11.7–2.52)	*0.202*	0.93 (−4.40–6.26)	0.729
SHBG[c]	51.9 (19.5)	−0.92 (−5.92–4.08)	0.714	4.01 (−4.91–12.9)	*0.372*	0.46 (−3.83–4.75)	0.832

NOTE: Linear Regression analysis. Adjusted for age, BMI, parity, smoking, OC. Regression coefficient and 95% CI.
Abbreviations: E_2, 17β-estradiol; HDL-C, high-density lipoprotein cholesterol; OC, oral contraceptive use.
[a]Numbers may vary due to missing information.
[b]Daily salivary samples throughout one entire menstrual cycle.
[c]Serum samples in early follicular phase: days 1 to 5 after onset of menstrual cycle.
[d]Serum samples in late follicular phase: days 7 to 12 after onset of menstrual cycle.
[e]Serum samples in luteal phase; days 21 to 25 after onset of menstrual cycle.
[f]Mammograms were taken at days 7 to 12 (mid-cycle phase) after onset of the menstrual cycle.

lowest HDL-C tertile having below-median absolute mammographic density, had a 41% higher overall average 17β-estradiol level ($P = 0.016$; Fig. 1A), and a 49% higher overall average progesterone level ($P = 0.017$; Fig. 1D). Similarly, women in the lowest HDL-C tertile, having above-median percent mammographic density, had a 50% higher average 17β-estradiol level ($P = 0.006$), compared with women in the lowest HDL-C tertile having below-median percent mammographic density (Fig. 1G).

The associations between HDL-C, in combination with salivary and serum estradiol and progesterone and absolute mammographic density, were studied by tertiles of HDL-C in multivariable analyses. In women with low HDL-C (<1.39 mmol/L), a one SD increase in mid-menstrual (β-value 3.99, $P = 0.041$) and follicular salivary 17β-estradiol (β value 4.67, $P = 0.014$), salivary luteal progesterone (β value 4.31, $P = 0.029$), and in AUC of progesterone (β value 4.36, $P = 0.026$) was associated with higher absolute mammographic density after adjustments (Table 3). No associations were found between serum or salivary estrogen and progesterone, and absolute mammographic density in women in middle and higher tertiles of HDL-C (Table 3).

The association between HDL-C, in combination with salivary and serum estradiol and progesterone, and percent mammographic density was also studied by tertiles of HDL-C in multivariable analyses (adjusted by age, BMI, parity, smoking habits, and previous OC-use). In women with low HDL-C (<1.39 mmol/L), a one SD increase in mid-menstrual 17β-estradiol (β value 3.15, $P = 0.032$) and follicular salivary 17β-estradiol (β value 3.77, $P = 0.008$) was both associated with a higher level of percent mammographic density. We also observed in women with high HDL-C (>1.67 mmol/L), that a one SD increase in mid-menstrual 17β-estradiol (β value 6.13, $P = 0.011$), and in follicular salivary 17β-estradiol (β value 6.05, $P = 0.014$), was associated with higher percent mammographic density (Table 4).

In stratified analysis by HDL-C (tertiles), we also studied the association between 17β-estradiol, progesterone, and above-median absolute mammographic density (>32.4 cm^2), and between 17β-estradiol, progesterone and above-median percent mammographic density (>28.5%). In women with low HDL-C (<1.39 mmol/L), a one SD increase of salivary 17β-estradiol in all menstrual phases was associated with 2.5 higher odds of having above-median absolute mammographic density (>32.4 cm^2; Table 5). Similar patterns were observed in women with low HDL-C (<1.39 mmol/L) between salivary 17β-estradiol in all menstrual phases and percent mammographic density (Table 5). Women with low HDL-C (<1.39 mmol/L), had by each SD increase in salivary 17β-estradiol in the mid-menstrual phase, a 4.12 (1.30–13.0) higher odds of having above-median percent mammographic density (>28.5%; Table 5).

No interactions were found between HDL-C tertiles and 17β-estradiol, whereas an interaction between salivary $AUC_{progesterone}$ and HDL-C was observed with absolute mammographic density ($P = 0.043$). No interactions were found between HDL-C and ovarian hormones with percent mammographic density (Table 5).

Discussion

In the present exploratory and hypothesis generating study, we observed in the subgroup of women with low HDL-C, a positive

Table 4. The associations between salivary and serum estradiol (SD) and progesterone (SD) and percent mammographic density (%), stratified by tertiles of HDL-C

		HDL-C <1.39 ($n = 66$)[a]		HDL-C 1.39–1.67 ($n = 68$)[a]		HDL-C >1.67 ($n = 65$)[a]	
Variables	**Mean (SD)**	**β-Coefficient (95% CI)**	***P***	**β-Coefficient (95% CI)**	***P***	**β-Coefficient (95% CI)**	***P***
Estradiol (E_2)							
Saliva, pmol/L[b]							
Mid-menstrual, days −7 to +6	18.2 (8.98)	3.15 (0.26–6.02)	0.032	−2.40 (−6.25–1.45)	0.218	6.13 (1.47–10.8)	0.011
Follicular phase, days −7 to −1	19.0 (9.58)	3.77 (1.01–6.53)	0.008	−2.68 (−6.96–1.60)	0.215	6.05 (1.27–10.8)	0.014
Luteal phase, days 0 to +6	17.4 (9.22)	2.10 (−0.86–5.07)	0.161	−2.03 (−5.56–1.51)	0.256	5.57 (1.01–10.1)	0.018
$AUC_{through\ cycle}$, time × pmol/L	269 (133)	3.14 (0.28–5.40)	0.032	−2.41 (−6.28–1.45)	0.217	6.12 (1.44–10.8)	0.011
Serum, nmol/L							
Early follicular[c]	0.15 (0.06)	−1.42 (−3.83–0.99)	0.242	−0.25 (−6.45–5.95)	0.936	3.72 (0.25–7.18)	0.036
Late follicular[d]	0.44 (0.31)	0.03 (−3.74–3.79)	0.989	−0.31 (−3.36–3.30)	0.985	0.21 (−3.63–4.05)	0.913
Late luteal[e]	0.43 (0.20)	2.08 (−0.94–5.11)	0.173	1.34 (−2.86–5.54)	0.526	4.29 (0.44–8.14)	0.030
Progesterone							
Saliva, pmol/L[b]							
Luteal, days 0 to +9	142 (73.5)	3.10 (0.18–6.02)	0.038	0.56 (−3.10–4.22)	0.760	3.66 (−1.12–8.44)	0.130
$AUC_{through\ cycle}$, time × pmol/L	1341 (718)	2.55 (−0.38–5.49)	0.086	0.20 (−3.54–3.95)	0.914	3.26 (−1.45–7.97)	0.171
Serum, nmol/L							
Early follicular[c]	4.83 (6.29)	−0.60 (−3.24–2.04)	0.650	−1.59 (−9.40–6.23)	0.686	0.06 (−3.02–3.15)	0.967
Late follicular[d]	5.24 (7.54)	1.60 (−2.20–5.39)	0.403	1.99 (−1.43–5.41)	0.250	3.85 (0.55–7.14)	0.023
Late luteal[e]	35.6 (20.1)	1.49 (−1.76–4.75)	0.363	−1.79 (−5.72–2.14)	0.366	1.78 (−2.26–5.82)	0.381
SHBG[c]	51.9 (19.5)	−0.28 (−4.01–3.46)	0.882	3.44 (−1.42–8.30)	0.162	0.41 (−2.86–3.68)	0.801

NOTE: Linear Regression analysis. Adjusted for age, BMI, parity, smoking, OC. Regression coefficient and 95% CI.

Abbreviations: E_2, 17β-estradiol; OC, oral contraceptive use.

[a]Numbers may vary due to missing information.

[b]Daily salivary samples throughout one entire menstrual cycle.

[c]Serum samples in early follicular phase: days 1–5 after onset of menstrual cycle.

[d]Serum samples in late follicular phase: days 7–12 after onset of menstrual cycle.

[e]Serum samples in luteal phase; days 21–25 after onset of menstrual cycle.

[f]Mammograms were taken at days 7–12 (mid-cycle phase) after onset of the menstrual cycle.

association between 17β-estradiol, progesterone, and both absolute and percent mammographic density. We observed among these women, a four times higher odds for having above-median percent mammographic density, and 2.5 times higher odds of having above-median absolute mammographic density for each one SD higher level of 17β-estradiol.

Recent observations linking obesity (37, 39), elevated cholesterol levels (3), low HDL-C (1, 29), and cholesterol metabolites (8) to breast cancer have provided new insights, but the association between HDL-C and mammographic density has been divergent (11, 26, 27, 40). Our findings of an association between HDL-C and mammographic density are supported by others (26), but few studies have reported on the association between hormones and mammographic density stratified by HDL-C levels. Interestingly, an inverse association between HDL-C and both absolute and percent mammographic density was recently observed restricted to women with low HDL-C levels (<50 mg/dl = 1.29 mmol/L; ref. 11), and supports our findings of an association between ovarian steroid hormones and mammographic density only among women with low HDL-C.

How to explain the U-shaped associations between HDL-C, estradiol and percent mammographic density in our study? It is challenging to study associations between breast cancer risk factors associated with obesity (i.e., low HDL-C) and mammographic density phenotypes, because obesity is inversely associated with percent density in particular (17, 18, 41, 42), but less prominent with respect to absolute mammographic density (18). Thus, we hypothesize that this may partly explain the U-shaped associations between HDL-C, estradiol and percent mammographic density in our study, reflecting residual confounding by BMI on percent mammographic density. Low HDL-C levels, which are linked to obesity, may vary by mammographic density phenotypes (27, 43). We also observed a higher inverse correlation between percent mammographic density and BMI compared with the correlation observed between absolute mammographic density and BMI, also supported by others (17, 18). An effect modification by BMI, on percent mammographic density in relation to breast cancer risk, has recently been suggested, as overweight women compared with normal weight women, had a somewhat higher breast cancer risk while having the same percent mammographic density (43).

Few previous studies have examined the association between HDL-C and mammographic density among groups of HDL-C levels, combined with endogenous estrogen and progesterone, and mammographic density phenotypes. Our findings, observed between estrogen and progesterone, and both absolute and percent mammographic density, only in women with low HDL-C levels, may reflect complex biologic processes. Low HDL-C and sex hormone levels may, in combination, stimulate growth of epithelial and stromal tissues, influencing both absolute and percent mammographic density. Low levels of HDL-C have been observed to induce higher levels of proinflammatory cytokines (6), and proinflammatory cytokines were recently found to induce higher local estradiol levels and cellular proliferation in the breast (44, 45), and to be associatied with percent mammographic density (46). Furthermore, hypercholesterolemia, strongly associated with low HDL-C, may induce angiogenesis (47), and accelerating breast cell growth and metastasis (8, 9).

The small HDL-C particles transporting excess cholesterol for excretion (4) have a wide variety of anti-inflammatory properties, and low HDL-C may fail to limit the level of proinflammatory cytokines (5–7). Thus, the breast tissue may experience higher levels of circulating cholesterol (8, 9),

Table 5. OR for having above-median absolute (>32.4 cm^2) and percent (>28.5%) mammographic density[e] per one SD of higher hormone levels stratified by tertiles of HDL-C

Variables	HDL <1.39 ($n = 66$)[a] OR (95% CI)	HDL 1.39–1.67 ($n = 68$)[a] OR (95% CI)	HDL >1.67 ($n = 65$)[a] OR (95% CI)	$P_{interaction}$[f]
Absolute density >32.4 cm^2				
17β-Estradiol				
Saliva[b]				
Mid-menstrual, days −7 to +6, pmol/L	2.48 (1.13–5.50)	0.67 (0.36–1.26)	1.42 (0.61–3.32)	0.205
Follicular phase, days −7 to −1, pmol/L	2.58 (1.19–5.56)	0.57 (0.28–1.19)	1.62 (0.65–4.01)	0.250
Luteal phase, days 0 to +6, pmol/L	2.03 (1.01–4.11)	0.77 (0.44–1.34)	1.23 (0.55–2.73)	0.219
$AUC_{through\ cycle}$, time × pmol/L	2.52 (1.13–5.60)	0.67 (0.35–1.26)	1.51 (0.64–3.59)	0.243
Serum				
Early follicular, nmol/L[c]	0.90 (0.55–1.46)	0.53 (0.20–1.36)	0.83 (0.45–1.54)	0.413
Progesterone				
Saliva[b]				
Luteal phase, days 0 to +9, pmol/L	1.94 (0.94–4.03)	0.97 (0.54–1.74)	1.03 (0.47–2.25)	0.067
$AUC_{through\ cycle}$, time × pmol/L	1.89 (0.92–3.90)	0.90 (0.49–1.63)	0.95 (0.45–2.03)	0.043
Serum				
Early follicular, nmol/L[c]	1.24 (0.78–1.95)	0.41 (0.08–2.08)	0.36 (0.06–2.22)	0.080
Late luteal, nmol/L[d]	1.20 (0.63–2.29)	0.64 (0.36–1.16)	0.97 (0.48–1.98)	0.508
Percent density >28.5%				
17β-Estradiol				
Saliva[b]				
Mid-menstrual, days −7 to +6, pmol/L	4.12 (1.30–13.0)	0.90 (0.45–1.82)	2.35 (0.59–9.44)	0.669
Follicular phase, days −7 to −1, pmol/L	4.55 (1.39–15.0)	0.70 (0.30–1.64)	2.06 (0.47–9.09)	0.507
Luteal phase, days 0 to +6, pmol/L	3.11 (1.15–8.43)	1.05 (0.57–1.96)	2.32 (0.65–8.33)	0.897
$AUC_{through\ cycle}$, time × pmol/L	4.26 (1.29–14.0)	0.89 (0.44–1.81)	2.50 (0.61–10.2)	0.700
Serum				
Early follicular, nmol/L[c]	0.44 (0.14–1.36)	0.74 (0.26–2.11)	3.25 (1.06–9.92)	0.073
Progesterone				
Saliva[b]				
Luteal phase, days 0 to +9, pmol/L	1.57 (0.76–3.24)	0.88 (0.45–1.72)	2.01 (0.52–7.82)	0.824
$AUC_{through\ cycle}$, time × pmol/L	1.48 (0.75–2.91)	0.76 (0.38–1.51)	1.92 (0.49–7.57)	0.863
Serum				
Early follicular, nmol/L[c]	0.49 (0.15–1.61)	0.51 (0.12–2.16)	1.10 (0.49–2.47)	0.333
Late luteal, nmol/L[d]	1.51 (0.71–3.21)	0.86 (0.43–1.70)	1.26 (0.54–2.90)	0.561

NOTE: Logistic regression analysis. Adjusted for BMI, age, number of children, smoking, previous use of oral contraceptives (OCs).
Abbreviation: E_2, 17β-estradiol.
[a]Numbers may vary due to missing information.
[b]Daily salivary samples throughout one entire menstrual cycle.
[c]Serum samples in early follicular phase: days 1to 5 after onset of menstrual cycle.
[d]Serum samples in luteal phase: days 21 to 25 after onset of menstrual cycle.
[e]Mammograms were taken at days 7 to 12 (mid-cycle phase) after onset of the menstrual cycle.
[f]Interaction: cross-product between hormones and tertiles of HDL-C.

increased low-grade inflammation (44), and higher levels of total endogenous estradiol and estradiol locally produced in the breast (44, 45). Moreover, immune cells and cytokines may interact in a paracrine manner with ovarian steroids in mammary cells (48), and support the present observation, and the hypothesis that mediators of inflammatory cellular cascades, such as low HDL-C, may influence mammographic density phenotypes (12).

Unfavorable metabolic profiles, such as high BMI/excess weight and weight gain, are risk factors for postmenopausal breast cancer development (39, 49), but the association between excess weight/weight gain and premenopausal breast cancer may vary by ethnisities and has not yet been clarified (50, 51). Thus, different metabolic traits like BMI and HDL during premenopausal years are possible risk factors for postmenopausal breast cancer, and may also be indicators of later breast cancer risk (49) through biomarkers such as mammographic density (11).

Our study combines several unique features. By having mammographic density measures, obtained at a standard time in the menstrual cycle, we avoid the bias of variation in mammographic density during the menstrual cycle (52). The validated, computer-assisted method quantifying the mammographic densities was read by one experienced blinded reader (14, 53). Endogenous estrogen and progesterone were assessed in both serum, and daily in saliva, throughout an entire menstrual cycle following strict validated methods (33), and at the same time during the menstrual cycle. This is the recommended approach, yet it is rarely achieved, due to its logistic complexity (54). This standardization enhanced the quality of our data and allowed the sampling of all clinical variables within the same narrow frame of the cycle for each participant. Furthermore, the variations in the length of the follicular phase may be greater than the variations in the luteal phase (55), but the second visit between days 7 and 12 of the menstrual cycle, and the third visit between days 21 and 25, should capture the late follicular phase and the luteal phase, respectively (55).

We also observed similar associations between late luteal serum estradiol and mammographic density phenotypes, compared with salivary estradiol measures. The study population was homogenous, including healthy women, and to limit any potential seasonal variation, women did not participate during the months with no daylight (December and January). Adherence to

the study was high, and all analyses and clinical examinations were conducted by the same trained personnel at one study site.

The present exploratory hypothesis generating study also had some disadvantages as our sample size was small, and the study design was cross-sectional. The small sample size, in combination with multiple testing, and the risk of false-positive results, support future research with a larger study population. However, our multiple salivary hormone variables are not considered to be independent measures, but indices within the same aligned menstrual cycle. Thus, multiple corrections with Bonferroni for each variable would be too stringent. Because of safety concerns, we could only obtain one measure of mammographic density, and therefore could not measure density pattern changes over a menstrual cycle. The assessment of daily salivary levels of unbound bioavailable estradiol and progesterone throughout a menstrual cycle is unique, but there is a need for further studies, as total serum hormones and free unbound salivary hormone levels are often correlated within individuals, while pooled data often show no significant correlations (33, 56). Immunoassay methods used in the present study have recently most often been replaced by LC/MS-MS, which compared with the immunoassay method, is a more efficient way of analyzing salivary hormones with higher specificity and sensitivity. However, previous studies on estradiol measurements, specifically, have shown a high correlation between MS and immunoassays of 0.969 (57).

To conclude, the findings in this exploratory and hypothesis generating study, link lower levels of HDL-C, alone and in combination with endogenous estrogen and progesterone, with both absolute and percent mammographic density. These results are supported by plausible biologic mechanisms linking HDL-C to breast cancer development. However, our small hypothesis generating study requires confirmation in larger studies to define the clinical implications of these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: V.G. Flote, A. Iversen, A.-S. Furberg, I. Thune
Development of methodology: V.G. Flote, I. Thune
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Ursin, P.T. Ellison, I. Thune
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.G. Flote, H. Frydenberg, A. Iversen, M.W. Fagerland, P.T. Ellison, T. Egeland, I. Thune
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