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J Nutr. 2020 Sep 1;150(9):2419-2428.

https://doi.org/10.1093/jn/nxaa168

Serum phospholipid fatty acids and mammographic density in premenopausal women

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Support

This study was funded by the Carlos III Institute of Health (AESI PI15CIII/0029). The article presents independent research. The views expressed are those of the authors and not necessarily those of the Carlos III Institute of Health.

Conflict of Interest and Funding Disclosure

The authors declare that they have no competing interests.

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Word count: 4320 words

Number of figures: 1

Number of tables: 3

Supplementary data: Supplemental Table 1

Running title

Serum fatty acids and mammographic density

List of abbreviations

BMI: body mass index

DHA: docosahexaenoic acid

EPA: eicosapentaenoic acid

FADS1: fatty acid desaturase 1

MD: mammographic density

MUFAs: monounsaturated fatty acids

PUFAs: polyunsaturated fatty acids

SCD-1: stearoyl-CoA desaturase 1

SFAs: saturated fatty acids

ABSTRACT

Background: The role of fatty acids (FAs) on mammographic density (MD) is unclear, and available studies are based on self-reported dietary intake.

Objective: This study assessed the association between specific serum phospholipid fatty acids (PL-FAs) and MD in premenopausal women.

Methods: The cross-sectional study DDM-Madrid recruited 1,392 Spanish premenopausal women, aged 39 to 50, who attended a screening in a breast radiodiagnosis unit of Madrid City Council. Women completed lifestyle and food frequency questionnaires. Percent MD was estimated using a validated computer tool (DM-Scan), and serum PL-FAs percentages were measured by gas chromatography–mass spectrometry (GC–MS). Multivariable linear regression models were used to quantify the association of FA tertiles with MD. Models were adjusted for age, education, body mass index, waist circumference, parity, oral contraceptives use, previous breast biopsies and energy intake, and corrected for multiple testing.

Results: Women in the third tertile of saturated FAs showed significantly higher MD compared to those in the first tertile (β_{T3vsT1}=7.53; 95%CI=5.44, 9.61). Elevated relative concentrations of palmitoleic (β_{T3vsT1}=3.12; 95%CI=0.99, 5.25) and gondoic ($\beta_{T3vsT1=}$2.67; 95%CI=0.57, 4.77) monounsaturated FAs, as well as high relative concentrations of palmitelaidic (β_{T3vsT1}=5.22; 95%CI=3.15, 7.29) and elaidic (β_{T3vsT1}=2.69; 95%CI=0.59, 4.79) *trans*-FAs, were also associated with higher MD. On the contrary, women with elevated relative concentrations of n-6 linoleic (β_{T3vsT1}=-5.49; 95%CI=-7.62, -3.35) and arachidonic (β_{T3vsT1}=-4.68; 95%CI=-6.79, -2.58) polyunsaturated FAs showed lower MD. Regarding desaturation indices, an elevated palmitoleic to palmitic ratio and a low ratio of oleic to steric and arachidonic to dihomo-γ-linolenic acids were associated with higher MD.

Conclusions: Spanish premenopausal women with high relative concentrations of most saturated FAs and some monounsaturated and *trans*-FAs showed an increased MD, while those with high relative concentrations of some n-6 polyunsaturated FAs presented lower density. These results, which should be confirmed in further studies, underscore the importance of analyzing serum FAs individually.

Keywords

Breast density, fatty acids, desaturation index, pre-menopause, DDM-Madrid, biomarkers, fat, breast cancer, epidemiology

Introduction

The mammographic image reflects variations in the composition of the breast tissue: the darker areas correspond to the fatty tissue, while the lighter or denser areas represent the fibroglandular tissue. The term "mammographic density" (MD) refers to the percentage of mammography composed of radiologically dense tissue, and it is an important risk factor for developing breast cancer (1,2). Given that mammographic density can be influenced by dietary factors, such as a Western dietary pattern, calorie intake or olive oil consumption (3,4), the identification of these specific nutrients may be of special interest for breast cancer prevention.

The role of fat intake on breast cancer risk has been widely investigated, but the evidence is too limited to draw any conclusion (5). Several studies have shown that some saturated fatty acids (SFAs) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are associated with a higher breast cancer risk, while omega-3 (n-3) PUFAs and the n-3/n-6 PUFA ratio seem to be protective, mainly in Asian populations (6,7). However, a meta-analysis of prospective cohort studies found no association with either fatty acid (FA) intake or serum FAs (8). More recently, elevated risk of breast cancer has been associated with high levels of circulating industrial *trans*-FAs in the European Prospective Investigation in Cancer and Nutrition (EPIC) study (9) and in the American Nurses' Health study-II (10).

In relation to MD, most studies have focused on the study of large groups of FAs, instead of analyzing their individual effect. The majority of these studies detected an association with SFAs (11–14) with the exception of one that detected an inverse association in premenopausal women (15). With respect to monounsaturated fatty acids (MUFAs), although Masala et al. detected lower MD with elevated intake of these FAs (16), most authors did not report any association (12–15,17,18). Regarding PUFAs, the results are more inconsistent. While some studies reported higher MD among premenopausal (15,16), or lower MD among postmenopausal women with elevated PUFA levels (17), others found no association (13,18). Finally, more recent studies showed that n-3 PUFAs might not be associated (19), or inversely associated with breast density among postmenopausal women (20,21) and in animal models (22,23), while n-6 PUFAs do not appear to be associated with MD (19,20).

The biological connection between serum FAs and MD, although not well established, could arise through inflammatory processes. There is evidence that some SFAs and PUFAs have anti-inflammatory or pro-inflammatory properties (24,25), and the expression of these inflammatory markers in normal breast tissue has also been associated with MD in pre and postmenopausal women (26). Fatty acids levels could also exert a direct causal effect on breast density, helping to increase / decrease the relative amount of non-dense fatty tissue in the breast and, therefore, decrease / increase MD. On the other hand, this association could also reflect the indirect influence that steroid hormones have on MD, since it has been observed that these hormones can modify the biosynthesis of unsaturated FAs, increasing the expression of stearoyl-CoA desaturase 1 (SCD-1) and decreasing the activity of the Δ5-desaturase and Δ**6**-desaturase enzymes (27). It is well known that small variations in endogenous sex hormones levels and insulin-like growth factors can also affect MD, even in premenopausal women (28,29)**.**

Epidemiological studies that have analyzed the association between FAs and MD have assessed FAs intake using dietary questionnaires. Only Hudson's study (19) assessed PUFAs in erythrocyte membranes. Blood FA levels can be used as biomarkers of diet and metabolic processes. Many essential n-3 PUFAs, n-6 PUFAs and *trans*-FAs that cannot be endogenously synthesized by humans, and that must be obtained from diet, are good biomarkers of dietary intake. On the other hand, SFAs and MUFAs can be synthesized de novo in humans, and therefore circulating levels do not necessarily represent diet (30). On the other hand, it has been described that self-reported dietary habits are prone to systematic and random measurement errors (31,32). Therefore, serum FA concentration may be a more accurate measure of the bioavailable amounts of these fats. The main objective of this study was to analyze the association between the relative concentrations of individual serum phospholipid fatty acids (PL-FAs) and MD in Spanish premenopausal women.

Methods

Study population

DDM-Madrid is a cross-sectional study based on 1,466 premenopausal women, aged 39 to 50, recruited between June 2013 and May 2015 at the Medical Diagnostic Centre of Madrid City Council (Madrid Salud), where these women went for their routine gynecological examination. The participation rate was 88%. Women were contacted by telephone and invited to participate. The same day that the women attended their medical examination, they signed a written informed consent and three interviewers interviewed them using

a standardized epidemiological questionnaire that has been previously used in the DDM-Spain study (33,34). This questionnaire contained sociodemographic information, data on childhood and youth, family and personal history, gynecological, obstetric and occupational history, smoking, alcohol and physical activity. Recreational physical activity during the previous year was assessed using a translation of a validated self-administered questionnaire that takes into account duration, frequency and intensity of 26 activities (35). Total metabolic equivalents (MET-h/week) were calculated according to the 2011 Compendium of Physical Activities (36). Finally, participants completed a 117-item semi-quantitative food frequency questionnaire, adapted and validated in several Spanish adult populations (37,38), which included the eating habits of the previous 12 months.

On the same day, the interviewers measured and weighed the participants using a certified scale. Body mass index (BMI) was estimated as the weight in kilograms divided by the square of height in meters. Following a standardized procedure, they also measured the women's waist and hip circumference. All these variables were measured twice, with a third measure if the first two were discrepant. The average anthropometric values were used in the analyses.

Interviewers also extracted a fasting blood sample from each woman, which was subsequently centrifuged, aliquoted and stored at -80° in the biobank of the Carlos III Institute of Health. DDM-Madrid study was conducted in accordance with the Declaration of Helsinki guidelines and was formally approved by the Ethics and Animal Welfare Committee of this Institute.

Mammographic density assessment

MD was assessed using 2D digital mammograms that workers undergo during their annual gynecological examination at the Madrid Salud center. The craniocaudal and mediolateral oblique views of the left and right breast mammograms of each woman were collected and anonymized. An experienced radiologist estimated the percentage of MD from the craniocaudal mammogram of the left breast assisted by DM-Scan, a free semi-automated computer tool that quantifies MD on digital mammograms, on a continuous scale and in DICOM format. This tool identifies the pixels that correspond to adipose tissue, dense tissue and the background of the image. Based on these values two thresholds are created that allow estimating the proportion that corresponds to the dense area of the breast. DM-Scan has shown a high reproducibility and validity (39,40). To assess the internal consistency of the radiologist, a sample of 100 mammograms were

read again, and an intra-class correlation coefficient of 0.87 (95%CI =0.82-0.92) between the first and second reading was obtained.

Protocol for analysis of PL-FAs

PL-FAs were determined by using the protocol proposed by Criado-Navarro et al. (41) which is based on the isolation of PLs using 30 mg HybridSPE® cartridges from Supelco (Bellefonte, PA, USA), derivatization of the resulting extract to convert PL-FAs into their more volatile PL-FAMEs, and GC–MS analysis. The NIST Mass Spectral Search Program v.11.0 (NIST, Washington, DC, USA) was used for spectral search (Mainlib and Replib libraries). Tentative identification was reported when the correlation between experimental and database spectra was above 0.75 in normal search mode. Confirmatory analysis was carried out by analysis of a FAMEs multistandard from Sigma–Aldrich.

Relative quantitation of PL-FAs

The relative concentration of each PL-FA, expressed as percentage of serum total PL-FAs, was quantified by integrating the area under the peak and dividing the result by the total PL-FA area. The variability of the determination, expressed as variation coefficient in percentage, ranged from 0.3 to 14.9%. A total of 21 individual PL-FAs belonging to the following classes were determined: SFAs (14:0, 15:0, 16:0, 17:0, 18:0 and 20:0), *cis*-MUFAs (16:1 n-7, 17:1, 18:1 n-9 and 20:1 n-9), ruminant *trans*-FAs (16:1 n-7 and 18:1 n-7), industrial *trans*-FAs (18:1 n-9), n-3 PUFAs (18:3, 20:5 and 22:6) and n-6 PUFAs (18:2, 18:3, 20:2, 20:3 and 20:4). Several desaturation indices were also calculated: the ratio between palmitoleic acid and palmitic acid (SCD-16 or DI_{16}) and the ratio between oleic acid and stearic acid (SCD-18 or DI_{18}), as biomarkers of the stearoyl-CoA desaturase 1 (SCD-1) (Δ9-desaturase) expression (42); the ratio of arachidonic to dihomo-γ-linolenic acid, indicator of the activity of the fatty acid desaturase 1 (FADS1) (Δ5-desaturase); and the ratio of dihomo-γ-linolenic acid to linoleic acid, indicator of the activity of Δ6-desaturase and elongase (43).

Statistical methods

After excluding women whose mammogram could not be read, women with analogical images, those whose relative concentrations of serum PL-FAs could not be measured, women who were not fasting when their blood was drawn and those with lacking information on the main confounding variables, the final sample size included 1,392 participants (95%).

Descriptive characteristics of the participants were summarized as absolute values and percentages. Mean MD levels, as well as mean relative concentrations of the main FA classes (SFAs, *cis*-MUFAs, *trans*-MUFAs, n-3 PUFAs and n-6 PUFAs) according to the women characteristics, were also described and compared using Wald test.

To analyze the association between MD and relative percentage of serum PL-FAs, the latter were divided into tertiles. The second and third tertiles were compared with the first tertile (reference) using multivariable linear regression models. Two linear models where fitted: the first was adjusted for age (continuous variable) and BMI (continuous variable). The second model was further adjusted for educational level (primary school or less, secondary school, university graduate) and those variables that were associated with MD ($p<0.05$): waist circumference (in tertiles), parity (nulliparous, 1, 2, >2 children), oral contraceptives use (never, past use, current use), previous breast biopsies (none, yes) and energy intake (continuous variable). The linear trend across tertiles was also tested with the Wald test. In addition to categorical analyses, each type of serum PL-FA was modelled through a restricted quadratic spline with knots at the 5th, 50th, and 95th percentiles (44). These restricted quadratic splines allowed two different quadratic trends on either side of the median PL-FA that were restricted to be linear below the 5th percentile and above the 95th percentile, so they could reproduce a large variety of smooth dose-response curves while avoiding implausible shapes at extreme relative concentrations of PL-FAs. As a sensitivity analysis, we conducted a subset analysis by BMI and waist circumference (women with BMI < 25Kg/m^2 and waist circumference $\leq$ 80 cm (721 women) versus women with BMI $\geq$ 25Kg/m^2 and waist circumference > 80 cm (396 women), and tests of heterogeneity of associations were carried out. To account for the problem of multiple testing, p-values were adjusted using the false discovery rate proposed by Benjamini & Hochberg (45). All analyses were performed using STATA/MP 14.2 software.

Results

The mean MD of the women was 34.3% (IQR: 21.9 - 46.8). The general characteristics of the study population and the distribution of MD according to these characteristics are presented in Table 1. Briefly, women's mean age at recruitment was 44 years. Most of them attended university, and one third was overweight or obese. Almost half of the participants had two children, and more than half ever used oral contraceptives, were none smokers and consumed less than 10 g of alcohol per day. The percentage of sedentary women was 42%, 7% had first-degree relatives with breast cancer, and 10% had previous breast

biopsies. The percentage of women who took statins to treat their hypercholesterolemia was 2%, and the mean caloric intake was 1968 ± 593 kcal/day. Women with higher BMI and larger waist circumference had significantly lower MD. Higher MD was observed among nulliparous women, in those who never used oral contraceptives, in participants with previous breast biopsies, and in those with higher caloric intake.

Table 2 shows the distribution of serum PL-FA according to women's characteristics. Relative concentrations of SFAs were higher in less educated women, with higher BMI, with larger waist circumference and among physically inactive women. Relative concentrations of *cis*-MUFAs were higher among university-educated women, in women with lower BMI and lower waist circumference, in those with higher alcohol consumption and among physically active women. With regard to relative *trans*-MUFA concentrations, these were also higher in women with higher education and lower BMI and waist circumference, but also in women with previous breast biopsies and with lower caloric intake. Younger and slimmer women, current users of oral contraceptives and women without hypercholesterolemia showed higher relative concentrations of n-6 PUFAs. Finally, older, university, nulliparous and physically active women, as well as those with higher alcohol and lower caloric intake were the participants with higher relative concentrations of n-3 PUFAs.

The association between individual FAs and MD is shown in Table 3. Women in the third tertile of all SFAs showed a significantly higher MD compared to those in the first tertile, with the exception of palmitic acid, for which an inverse association was observed (β_{T3vsT1}=-2.29; 95%CI=-4.43, -0.15; *P*-trend=0.058). Regarding *cis*-MUFAs, we observed a significant association with palmitoleic, heptadecenoic and gondoic acids. However, no association was detected with total *cis*-MUFAs. The ruminant *trans*-palmitelaidic acid (β_{T3vsT1}=5.22; 95%CI=3.15, 7.29; *P*-trend=0.001) and the industrial *trans*-elaidic acid (β_{T3vsT1}=2.69; 95%CI=0.59, 4.79; *P*-trend=0.022) were also associated with higher MD. With regard to n-6 PUFAs, while high relative concentrations of γ-linolenic acid were associated with increased MD, women with elevated relative concentrations of linoleic acid and arachidonic acid showed lower density values. Therefore, the joint association of this subgroup turned out to be inverse and significant (β_{T3vsT1}=-7.68; 95%CI=-9.74, -5.62; *P*-trend=0.001). Finally, although no significant association was found between MD and n-3 PUFAs, the n-6/n-3 PUFA ratio showed a negative trend (β_{T3vsT1}=-2.52; 95%CI=-4.64, -0.39; *P*-trend=0.033). Regarding desaturation indices, a high ratio of palmitoleic acid to palmitic acid (SCD-16) was associated with higher MD, while the ratio of oleic acid to stearic acid (SCD-18) and the ratio between arachidonic and dihomo-γ-linolenic acids showed an inverse association.

Figure 1 shows the adjusted mean differences in MD for SFAs, *cis*-MUFAs, *trans*-MUFAs, n-3 PUFAs, n-6 PUFAs and the log-transformed n-6 to n-3 PUFA ratio. MD increased as the relative concentrations of serum SFAs increased, while MD decreased as the relative concentrations of n-6 PUFAs increased.

Analysis by BMI and waist circumference revealed no substantial difference in the association between individual FAs, FA groups, desaturation indices and MD (Supplemental Table 1). It is only worth noting that the association with total SFAs was slightly higher in obese women.

Discussion

This study aims to analyze the association between the relative concentrations of individual PL-FAs and percentage of DM in almost 1,400 premenopausal women attending the breast radiodiagnosis unit of Madrid City Council. Our results show that high relative concentrations of several serum SFAs are associated with higher MD, while elevated relative n-6 PUFA concentrations, mainly linoleic acid and arachidonic acid, are associated with lower MD values.

In Spain, more than 70% of SFAs intake comes from the almost equally distributed consumption of meat, dairy, oil and fat products (46). However, long-term intake of SFAs does not appear to correlate well with blood levels, since these can be synthesized endogenously (30). It is worth noting that the relative concentration of serum SFAs in our participants (55%) is considerably higher than that detected in another previous study (40%) (47). Our results show that women with high relative concentrations of SFAs presented higher MD. An association with breast cancer risk has already been suggested by several case-control and cohort studies (6). However, their association with MD has been less studied. The SFA most strongly associated with MD was arachidic acid. In a recent nested breast cancer case-control study of premenopausal women, a statistical interaction with BMI was also found for this FA, with lower breast cancer risk found among women with BMI<25 kg/m^2 and higher risk among overweight/obese women (10). However, this interaction was not observed in our study. On the contrary, and in line with what was observed in our non-obese participants, these same authors observed an inverse association with palmitic acid (10), the most abundant SFA in serum.

MUFAs are biosynthesized from SFAs by the action of the enzyme SCD-1 in the liver, but they are also present in various foods. In Spain, oleic acid constitutes the most abundant MUFA, present in large quantities in olive oil (37% of the total MUFAs provided by the diet); but MUFAs are also present in meat products, pastries, precooked foods and in other products in less quantity (46). Although we did not detect an association between this group of FAs and MD, we observed a higher MD associated with palmitoleic acid among non-obese women. This is a n-7 MUFA biosynthesized from palmitic acid by the action of the enzyme SCD-1 in the liver, and high plasma levels of this FA have been associated with an increased breast cancer risk (9).

We also found higher MD associated with *trans*-palmitelaidic and *trans*-elaidic acids in non-obese women. Elaidic acid is formed during the partial hydrogenation of vegetable oils and it is found in a wide variety of industrial foods. Previous studies have described an association with total breast cancer (10,48), and with estrogen receptor negative tumors in particular (9). Elaidic acid has also been linked to lower risk of weight loss (49). Palmitelaidic acid is produced from biohydrogenation by bacteria in the rumen of ruminants, and consequently it is present in high-fat dairy products and meat of ruminants. Elevated blood levels of this *trans* FA have also been associated with higher breast cancer risk (10,48). While Hirko et al. (10) detected higher breast cancer risk only among obese women, we observed higher MD only among non-obese women. This discrepancy may be due to the fact that BMI behaves in the opposite way with these two endpoints: while it increases the risk of breast cancer in postmenopausal women, it is inversely associated with MD.

Another relevant finding of our study is the inverse association detected between MD and total n-6 PUFAs, mainly due to the lower MD associated with the two most common n-6 PUFAs: linoleic and arachidonic acids. These associations have not been detected in two previous studies (19,20). One of them detected that a high n-6/n-3 PUFA ratio was associated with a higher MD (20), and the other found a trend toward increased percent density with increased arachidonic acid (19). Regarding linoleic acid and breast cancer risk, while one meta-analysis concluded that high serum levels of this FA were associated with non-significant lower risk (36) (50), another suggested that the results from previous studies are too inconsistent to support this hypothesis (51). On the other hand, Sakai et al., in a systematic review on arachidonic acid and cancer risk, concluded that this PUFA was not associated with breast tumors (52). The inverse association detected with MD in our study is difficult to explain. Both PUFAs are linked together through

279 metabolism, since arachidonic acid is obtained by desaturation and chain elongation of linoleic acid, an
280 essential FA found in vegetable oils, nuts, and fatty seeds (53).
281
282 Regarding n-3 PUFAs, although these FAs seem to have an inhibiting role in the development and
283 progression of breast cancer (7), their association with MD is less conclusive. Some studies have detected
284 an inverse association between eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake and
285 MD among postmenopausal women (20,21), while others have not found such an association (54). Hudson
286 et al. also found no association between the concentration of these PUFAs in erythrocyte membranes and
287 percent density or dense breast area (19). The few studies that have analyzed premenopausal women, have
288 either detected a modest median decrease in absolute breast density (21) or, in line with our results, have
289 not detected any association (20). In any case, it is important to highlight the low relative serum
290 concentrations of n-3 PUFAs detected in the women under study. Their relative concentrations of EPA +
291 DHA are below the phospholipid concentrations reported for other European countries (55). This leads to a
292 n-6/n-3 PUFA ratio, much higher than the dietary ratio estimated in other large Spanish studies (56).
293
294 High SCD-16 and SCD-18 desaturation indices were associated with higher and lower MD, respectively.
295 Both indices reflect hepatic SCD-1 activity/expression, which converts SFAs to MUFAs, and they are
296 biomarkers of endogenous lipogenesis (42). Several epidemiological studies have described an association
297 between a high blood SFA/MUFA ratio, indicating low SCD-1 activity, and lower breast cancer risk,
298 suggesting that a reduction in the activity and expression of this enzyme in the liver could decrease the risk
299 of developing this tumor (42). Our results show opposing associations of SCD-16 and SCD-18 indices with
300 MD. Only the SCD-16 desaturation index has been associated with breast cancer risk in previous studies (9).
301 Although FA desaturation indices accurately reflect the activity of the enzyme (endogenous synthesis), other
302 environmental factors, such as the intake of other FAs, may influence these ratios. In this sense, given that
303 the dietary content of palmitoleic acid is lower than the oleic acid content, several authors claim that the
304 SCD-16 index could be a better marker of hepatic SCD-1 activity than the SCD-18 index (42,57,58). High
305 carbohydrate diets, insulin and estrogen levels, alcohol, or exercise training are other environmental factors
306 that can modify the FA desaturation indices (42). Regarding MD, only one previous study found an
307 association between decreasing levels of SCD-16 and SCD-18 and a progressive reduction in breast
308 density, but only among obese women (59). When we analyzed these associations by BMI and waist
309 circumference, we observed a stronger association with the SCD-16 index among non-obese women.

We also detected an inverse association between FADS1 and MD. This ratio is an indicator of the Δ5-desaturase activity, an enzyme encoded by the FADS1 gene that converts dihomo-γ-linolenic acid to arachidonic acid. Although alterations in Δ5-desaturase activity have been associated with various diseases, these do not seem to influence the development of breast cancer (43). Only two previous studies have observed a statistically significant (60) or borderline (61) association with this tumor.

Hudson et al. (19) previously assessed the association between circulating erythrocyte n-6 and n-3 PUFA levels and MD in 248 postmenopausal women. Therefore, this is the first study to date that explores the association of relative serum concentrations of individual SFAs, MUFAs and PUFAs with MD in premenopausal women. One of the main strengths of the study is the high number of women included and the high participation rate. All mammograms of the participants were done in the same center and with the same equipment. MD was measured on continuous scale using a validated computer-assisted method and by a single reader that showed high internal consistency. Another important strength is the wide range of FAs measured in serum phospholipids. Furthermore, compared to traditional self-reported assessment methods, which are more prone to measurement errors (31,32), this biomarker can provide a more objective estimate of the intake of those FAs that cannot be synthesized endogenously, such as some PUFAs and *trans*-FAs.

This study also has several limitations. First, the cross-sectional design did not allow us to determine temporal associations. Second, serum PL-FAs were evaluated only once, and although only fasting samples were used, their relative presence fluctuates with changes in dietary habits. However, although there are other biological specimens such as adipose tissue, more suitable to reflect long-term dietary intake, the FA composition of serum phospholipids is considered a convenient alternative in epidemiological studies (62). Third, although we adjusted the models by all established predictors, unmeasured residual confounders, associated with relative PL-FA concentrations (such as triglycerides, cholesterol, insulin or other dietary factors) or with MD (such as time of the menstrual cycle), may have interfered with the detected associations. Fourth, although unlikely, women with previous breast biopsies could have modified their dietary habits, resulting in a reverse causation bias. For this reason, a sensitivity analysis excluding these women was carried out, and the results were very similar to those obtained using the entire sample (data not shown). Fifth, to assess whether the outliers may have influenced our results, a second sensitivity analysis

was performed eliminating the most extreme density values. Although the estimators were slightly attenuated, no differences were observed with the associations detected in Table 3 (data not shown). Sixth, given the large number of test performed, we cannot rule out the possibility of some of the results being detected by chance. However, we statistically accounted for multiple comparisons using the Benjamini correction (45). Finally, although we have analyzed 21 individual FAs, the serum concentrations of most of them are very low. For this reason, we focused the discussion on those most abundant, which may have greater clinical relevance.

In conclusion, this study shows that relative concentrations of most SFAs, some MUFAs, as well as γ-linolenic acid were associated with higher MD, while high relative concentrations of palmitic, linoleic and arachidonic acids were associated with lower breast density. A low endogenous synthesis of palmitoleic acid and a high endogenous production of oleic and arachidonic acids were also associated with lower MD. This study emphasizes the importance of analyzing the association with serum PL-FAs individually. Given that this is an exploratory analysis, and that there are hardly any previous studies that have analyzed these associations, our results should be taken with caution and confirmed in future studies.

Acknowledgments

The authors' responsibilities were as follows: VL, MP, BPG, IR and VC: designed and conducted the research; ICN, PL, AS, DST, RL, IM and FPC: provided essential materials; VL, MPP, RPB, ER, AC and MP: analyzed the data; VL, MPP and MP: wrote the paper. VL: had primary responsibility for the final content; and all authors: revised the manuscript critically for important intellectual content, and read and approved the final manuscript.

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Table 1. Descriptive characteristics of premenopausal women and mammographic density percentage according to women's characteristics[1]

Charactersistic	n (%)	%MD	*P*-value[2]
Total	1392 (100.0)	34.3 ± 17.5	
Age, y			0.303
<45	747 (53.7)	35.5 ± 17.4	
≥45	645 (46.3)	33.0 ± 17.5	
Educational level			0.435
Primary school or less	63 (4.5)	30.7 ± 17.4	
Secondary school	481 (34.6)	32.8 ± 17.0	
University graduate	848 (60.9)	35.5 ± 17.7	
Body mass index, kg/m^2			<0.001
<18.5	24 (1.7)	45.1 ± 18.5	
18.5-24.9	914 (65.7)	38.8 ± 16.7	
25-29.9	318 (22.8)	27.2 ± 14.7	
≥30	136 (9.8)	19.0 ± 13.4	
Waist circumference[3], cm			<0.001
<74.35	461 (33.1)	43.7 ± 16.5	
74.35-83.05	456 (32.8)	34.4 ± 15.9	
>83.05	456 (32.8)	24.8 ± 14.5	
Unknown	19 (1.4)		
Number of children			0.001
None	333 (23.9)	37.2 ± 18.4	
1	326 (23.4)	34.8 ± 18.5	
2	654 (47.0)	33.0 ± 16.5	
>2	79 (5.7)	31.8 ± 15.7	
Use of oral contraceptives			0.022
Never	529 (38.0)	36.1 ± 18.4	
Past use	807 (58.0)	33.5 ± 16.9	
Current use	45 (3.2)	30.9 ± 15.4	
Unknown	11 (0.8)		
Tobacco consumption			0.192
None	533 (38.3)	35.5 ± 18.0	
Former smoker	484 (34.8)	33.8 ± 16.9	
Current smoker	375 (26.9)	33.3 ± 17.3	
Alcohol consumption, g/day			0.782
None	251 (20.3)	34.1 ± 17.5	
< 10	810 (65.6)	34.9 ± 17.4	
≥10	174 (14.1)	35.2 ± 17.0	
Physical activity, MET-h/week			0.753
None	576 (41.6)	32.9 ± 17.0	
≤12	345 (24.9)	34.0 ± 17.2	
>12	465 (33.5)	36.4 ± 18.0	
Family history of breast cancer			0.759
None	1085 (77.9)	34.2 ± 17.2	
Second degree only	210 (15.1)	34.9 ± 18.5	
First degree	97 (7.0)	34.3 ± 17.9	
Previous breast biopsies			<0.001
None	1247 (89.6)	33.5 ± 17.3	
Yes	145 (10.4)	41.6 ± 17.5	
Hypercholesterolemia			0.808
No	1196 (86.9)	34.5 ± 17.5	
Yes, not treated	150 (10.9)	34.5 ± 18.0	
Treated with statins	31 (2.3)	28.5 ± 13.8	
Total energy intake[3], kcal/day			0.011
<1673	409 (29.4)	33.5 ± 17.5	

1673-2151	408 (29.3)	35.8 ± 17.2
>2151	408 (29.3)	35.3 ± 17.2
Unknown[4]	167 (12.0)	

[1] Values are number of women (%) and means ± SDs. MD, mammographic density.
[2] P values adjusted for age and body mass index.
[3] In tertiles.
[4] Participants who did not answer the food frequency questionnaire.

Table 2. Mean percent of serum phospholipid fatty acids according to premenopausal women's characteristics [1]

		SFAs		cis-MUFAs		*trans*-FAs		n-6 PUFAs		n-3 PUFAs	
	n	% of Total PL-FAs	*P*-value	% of Total PL-FAs	*P*-value	% of Total PL-FAs	*P*-value	% of Total PL-FAs	*P*-value	% of Total PL-FAs	*P*-value
TOTAL	1392	54.80 ± 4.93		9.38 ± 2.25		1.43 ± 0.28		30.81 ± 4.22		3.57 ± 1.28	
Age, y			0.211		0.690		0.657		0.028		0.003
<45	747	54.64 ± 5.05		9.41 ± 2.48		1.43 ± 0.28		31.04 ± 4.27		3.48 ± 1.24	
≥45	645	54.98 ± 4.79		9.36 ± 1.95		1.43 ± 0.29		30.55 ± 4.14		3.69 ± 1.32	
Educational level			0.003		0.003		0.020		0.488		0.001
Primary school or less	63	56.24 ± 5.03		8.88 ± 2.20		1.39 ± 0.29		30.24 ± 4.33		3.26 ± 1.18	
Secondary school	481	55.07 ± 4.44		9.22 ± 1.89		1.41 ± 0.26		30.83 ± 3.78		3.46 ± 1.26	
University graduate	848	54.54 ± 5.16		9.51 ± 2.42		1.44 ± 0.29		30.85 ± 4.44		3.66 ± 1.29	
Body mass index, kg/m^2			<0.001		<0.001		<0.001		0.015		0.702
<18.5	24	53.13 ± 4.17		10.2 ± 1.61		1.54 ± 0.21		31.87 ± 4.27		3.26 ± 1.18	
18.5-24.9	914	54.45 ± 4.78		9.58 ± 2.08		1.45 ± 0.27		30.94 ± 4.07		3.58 ± 1.30	
25-29.9	318	55.18 ± 5.38		9.09 ± 2.77		1.41 ± 0.29		30.68 ± 4.81		3.63 ± 1.31	
≥30	136	56.52 ± 4.51		8.65 ± 1.81		1.30 ± 0.29		30.10 ± 3.54		3.43 ± 1.08	
Waist circumference[2], cm			0.025		<0.001		0.001		0.953		0.811
<74.3	461	54.49 ± 4.45		9.72 ± 2.10		1.45 ± 0.26		30.76 ± 4.00		3.58 ± 1.36	
74.3-83.0	456	54.64 ± 4.79		9.35 ± 1.83		1.45 ± 0.27		30.99 ± 3.92		3.57 ± 1.26	
>83.0	456	55.22 ± 5.49		9.09 ± 2.70		1.39 ± 0.30		30.74 ± 4.71		3.56 ± 1.24	
Number of children			0.073		0.175		0.068		0.788		<0.001
None	333	54.18 ± 4.53		9.54 ± 2.60		1.47 ± 0.28		30.92 ± 3.96		3.89 ± 1.44	
1	326	55.24 ± 5.28		9.33 ± 2.27		1.41 ± 0.30		30.47 ± 4.19		3.54 ± 1.29	
2	654	54.84 ± 4.99		9.35 ± 2.11		1.42 ± 0.27		30.93 ± 4.42		3.46 ± 1.18	
>2	79	55.18 ± 4.42		9.20 ± 1.55		1.45 ± 0.28		30.82 ± 3.61		3.35 ± 1.12	
Use of oral contraceptives			0.153		0.445		0.145		0.042		0.885
Never	529	55.01 ± 4.88		9.40 ± 2.01		1.41 ± 0.28		30.61 ± 4.08		3.57 ± 1.38	
Past use	807	54.64 ± 4.95		9.39 ± 2.43		1.44 ± 0.28		30.94 ± 4.27		3.59 ± 1.23	
Current use	45	54.40 ± 5.38		8.87 ± 1.47		1.43 ± 0.31		31.88 ± 4.64		3.42 ± 1.00	
Tobacco consumption			0.859		0.484		0.430		0.271		<0.001
None	533	54.92 ± 5.31		9.29 ± 2.44		1.43 ± 0.29		30.62 ± 4.55		3.74 ± 1.42	
Former smoker	484	54.58 ± 4.57		9.49 ± 2.17		1.43 ± 0.28		30.96 ± 3.96		3.54 ± 1.18	
Current smoker	375	54.90 ± 4.82		9.38 ± 2.06		1.42 ± 0.27		30.91 ± 4.04		3.40 ± 1.17	
Alcohol consumption, g/day			0.270		0.022		0.996		0.529		0.025
None	251	55.01 ± 4.95		9.24 ± 2.25		1.42 ± 0.28		30.84 ± 4.09		3.48 ± 1.26	
< 10	810	54.83 ± 4.98		9.35 ± 2.00		1.44 ± 0.28		30.75 ± 4.24		3.62 ± 1.30	
≥10	174	54.46 ± 4.61		9.78 ± 3.17		1.41 ± 0.25		30.57 ± 4.10		3.77 ± 1.29	

Physical activity, MET-h/week			0.003		0.038		0.005		0.203		0.002
None	576	55.33 ± 5.27		9.16 ± 2.20		1.40 ± 0.29		30.66 ± 4.51		3.45 ± 1.18	
≤12	345	54.38 ± 4.80		9.70 ± 2.76		1.46 ± 0.28		30.82 ± 3.99		3.63 ± 1.27	
>12	465	54.43 ± 4.54		9.43 ± 1.83		1.45 ± 0.27		31.00 ± 4.01		3.69 ± 1.40	
Family history of breast cancer			0.336		0.870		0.461		0.177		0.239
None	1085	54.71 ± 4.90		9.41 ± 2.16		1.43 ± 0.28		30.89 ± 4.23		3.55 ± 1.28	
Second degree only	210	55.17 ± 5.04		9.13 ± 1.67		1.43 ± 0.29		30.61 ± 4.13		3.65 ± 1.28	
First degree	97	54.93 ± 5.06		9.61 ± 3.75		1.40 ± 0.28		30.39 ± 4.27		3.66 ± 1.30	
Previous breast biopsies			0.428		0.308		0.022		0.871		0.818
None	1247	54.83 ± 4.99		9.36 ± 2.26		1.42 ± 0.28		30.81 ± 4.24		3.57 ± 1.28	
Yes	145	54.49 ± 4.42		9.56 ± 2.11		1.48 ± 0.28		30.87 ± 3.99		3.60 ± 1.28	
Hypercholesterolemia			0.256		0.588		0.173		0.030		0.115
No	1196	54.76 ± 5.00		9.35 ± 2.06		1.42 ± 0.28		30.92 ± 4.30		3.55 ± 1.27	
Yes, not treated	150	54.91 ± 4.34		9.43 ± 2.07		1.48 ± 0.26		30.44 ± 3.47		3.74 ± 1.35	
Treated with statins	31	55.92 ± 4.65		9.48 ± 2.86		1.41 ± 0.28		29.51 ± 3.17		3.67 ± 1.34	
Total energy intake[2], kcal/day			0.077		0.153		0.026		0.885		0.001
<1673	409	54.50 ± 4.99		9.50 ± 1.94		1.45 ± 0.28		30.78 ± 4.14		3.77 ± 1.36	
1673-2151	408	54.86 ± 4.58		9.40 ± 2.20		1.44 ± 0.26		30.69 ± 3.70		3.61 ± 1.27	
>2151	408	55.11 ± 5.16		9.27 ± 2.57		1.41 ± 0.28		30.74 ± 4.65		3.47 ± 1.25	

[1] Values are mean percentages ± SDs. MUFAs, monounsaturated fatty acids; n-3 PUFAs, omega-3 polyunsaturated fatty acids; n-6 PUFAs, omega-6 polyunsaturated fatty acids; PL-FAs, phospholipid fatty acids; SD, standard deviation; SFAs, saturated fatty acids.
[2] In tertiles.

Table 3. Difference in mammographic density percentage in premenopausal women by tertiles of serum phospholipid fatty acids[1]

		Model 1[2]			Model 2[3]		
		Tertile 2	Tertile 3		Tertile 2	Tertile 3	
Fatty acids	% of Total PL-FAs	β (95%CI)	β (95%CI)	*P*-trend[4]	β (95%CI)	β (95%CI)	*P*-trend[4]
SFAs							
14:0 myristic acid	0.20 ± 0.16	7.19 (5.23, 9.14)	6.30 (4.32, 8.29)	0.001	6.79 (4.76, 8.81)	6.94 (4.84, 9.05)	0.001
15:0 pentadecanoic acid	0.09 ± 0.05	5.00 (3.03, 6.98)	4.68 (2.70, 6.65)	0.001	6.10 (4.05, 8.15)	6.20 (4.13, 8.26)	0.001
16:0 palmitic acid	32.91 ± 1.98	0.38 (-1.61, 2.38)	-2.03 (-4.04, -0.02)	0.091	0.67 (-1.41, 2.76)	-2.29 (-4.43, -0.15)	0.060
17:0 margaric acid	0.26 ± 0.46	3.72 (1.74, 5.70)	3.88 (1.90, 5.87)	0.001	3.99 (1.90, 6.08)	4.77 (2.69, 6.84)	0.001
18:0 stearic acid	21.21 ± 4.33	4.84 (2.88, 6.79)	8.13 (6.17, 10.10)	0.001	4.99 (2.94, 7.04)	7.71 (5.63, 9.78)	0.001
20:0 arachidic acid	0.12 ± 0.06	8.02 (6.11, 9.93)	10.53 (8.61, 12.44)	0.001	8.22 (6.23, 10.20)	10.73 (8.73, 12.73)	0.001
Total SFAs	54.80± 4.93	6.40 (4.45, 8.35)	7.77 (5.80, 9.73)	0.001	6.80 (4.76, 8.83)	7.53 (5.44, 9.61)	0.001
MUFAs							
16:1 n-7 palmitoleic acid	0.32 ± 0.18	1.91 (-0.08, 3.90)	1.92 (-0.09, 3.93)	0.109	2.39 (0.32, 4.46)	3.12 (0.99, 5.25)	0.009
17:1 heptadecenoic acid	0.02 ± 0.12	2.05 (0.06, 4.04)	3.62 (1.63, 5.60)	0.001	3.28 (1.21, 5.35)	4.95 (2.87, 7.03)	0.001
18:1 n-9 oleic acid	8.97 ± 2.19	-1.42 (-3.43, 0.59)	-0.73 (-2.77, 1.32)	0.582	-1.35 (-3.45, 0.76)	-0.81 (-2.95, 1.33)	0.553
20:1 n-9 gondoic acid	0.07 ± 0.03	3.22 (1.22, 5.22)	1.69 (-0.32, 3.70)	0.155	3.87 (1.78, 5.97)	2.67 (0.57, 4.77)	0.024
Total cis-MUFAs	9.38 ± 2.25	-1.12 (-3.12, 0.89)	-0.74 (-2.78, 1.29)	0.582	-0.97 (-3.07, 1.12)	-0.71 (-2.84, 1.43)	0.574
Trans-fatty acids							
Ruminant trans-fatty acids							
16:1 n-7 palmitelaidic acid	0.13 ± 0.05	2.53 (0.55, 4.51)	4.40 (2.41, 6.39)	0.001	3.57 (1.50, 5.65)	5.22 (3.15, 7.29)	0.001
18:1 n-7 vaccenic acid	1.16 ± 0.24	1.07 (-0.94, 3.08)	-1.56 (-3.58, 0.45)	0.175	1.30 (-0.81, 3.42)	-1.06 (-3.18, 1.06)	0.391
Total Ruminant trans-fatty acids	1.29±0.26	1.96 (-0.05, 3.96)	-1.36 (-3.37, 0.66)	0.243	2.55 (0.45, -4.65)	-0.89 (-3.00, -1.22)	0.471
Industrial trans-fatty acids							
18:1 n-9 elaidic acid	0.14 ± 0.06	2.17 (0.18, 4.16)	1.82 (-0.17, 3.82)	0.127	2.38 (0.30, 4.46)	2.69 (0.59, 4.79)	0.022
n-6 PUFAs							
18:2 linoleic acid	19.62 ± 3.69	-1.14 (-3.13, 0.84)	-4.67 (-6.70, -2.65)	0.001	-1.61 (-3.70, 0.48)	-5.49 (-7.62, -3.35)	0.001
18:3 γ-linolenic acid	0.05 ± 0.04	3.59 (1.61, 5.58)	3.91 (1.92, 5.90)	0.001	3.50 (1.42, 5.59)	3.77 (1.69, 5.85)	0.001
20:2 eicosadienoic acid	0.15 ± 0.05	-0.42 (-2.42, 1.57)	0.38 (-1.62, 2.37)	0.786	0.70 (-1.39, 2.78)	1.93 (-0.16, 4.03)	0.099
20:3 dihomo-γ-linolenic acid	2.15 ± 0.67	-1.33 (-3.32, 0.67)	-3.76 (-5.84, -1.69)	0.001	0.08 (-2.00, 2.16)	-1.33 (-3.57, 0.91)	0.340
20:4 arachidonic acid	8.84 ± 1.92	-3.73 (-5.71, -1.76)	-6.35 (-8.33, -4.38)	0.001	-2.84 (-4.93, -0.76)	-4.68 (-6.79, -2.58)	0.001
Total n-6 PUFAs	30.81 ± 4.22	-2.66 (-4.61, -0.71)	-8.07 (-10.03, -6.11)	0.001	-2.28 (-4.32, -0.24)	-7.68 (-9.74, -5.62)	0.001
n-3 PUFAs							
18:3 α-linolenic acid	0.04 ± 0.03	2.02 (0.02, 4.01)	0.88 (-1.14, 2.89)	0.504	2.09 (0.02, 4.16)	1.95 (-0.17, 4.06)	0.096
20:5 eicosapentaenoic acid (EPA)	0.64 ± 0.56	0.74 (-1.26, 2.74)	0.33 (-1.68, 2.33)	0.802	0.76 (-1.34, 2.86)	0.61 (-1.50, 2.72)	0.611
22:6 docosahexaenoic acid (DHA)	2.90 ± 0.88	1.19 (-0.80, 3.19)	0.06 (-1.94, 2.06)	0.950	0.84 (-1.26, 2.94)	0.51 (-1.61, 2.63)	0.654
Total n-3 PUFAs	3.57 ± 1.28	1.85 (-0.14, 3.84)	0.23 (-1.77, 2.22)	0.845	1.65 (-0.45, 3.75)	0.47 (-1.65, 2.58)	0.669

n-6/n-3 PUFA ratio [5]	9.12 ± 1.51	0.48 (-1.51, 2.47)	-2.52 (-4.52, -0.53)	0.029	0.47 (-1.63, 2.56)	-2.52 (-4.64, -0.39)	0.033
Desaturation indices							
SCD-16: 16:1n-7c/16:0	0.01 ± 1.57	1.36 (-0.63, 3.35)	1.72 (-0.29, 3.72)	0.143	2.00 (-0.07, 4.07)	2.94 (0.83, 5.06)	0.012
SCD-18: 18:1n-9c/18:0[5]	0.42 ± 1.42	-1.70 (-3.69, 0.29)	-5.36 (-7.37, -3.35)	0.001	-0.82 (-2.91, 1.27)	-4.85 (-6.96, -2.74)	0.001
FADS1: 20:4n-6/20:3n-6[5]	4.21 ± 1.38	-1.90 (-3.91, 0.11)	-1.77 (-3.81, 0.28)	0.143	-1.89 (-4.02, 0.24)	-2.87 (-5.00, -0.73)	0.017
FADS2: 20:3n-6/18:2[5]	0.11 ± 1.48	0.04 (-1.97, 2.06)	-0.53 (-2.64, 1.58)	0.716	1.04 (-1.04, 3.13)	1.51 (-0.76, 3.78)	0.252

[1] Values are mean percentages ± SDs and mean differences in the percentage of mammographic density comparing tertile 2 and tertile 3 with tertile 1 (reference), n=1196. CI, confidence interval; MUFAs, monounsaturated fatty acids; n-3 PUFAs, omega-3 polyunsaturated fatty acids; n-6 PUFAs, omega-6 polyunsaturated fatty acids; PL-FAs, phospholipid fatty acids; SD, standard deviation; SFAs, saturated fatty acids.

[2] Adjusted for age and body mass index.

[3] Adjusted for age, educational level, body mass index, waist circumference, parity (with category of nulliparous), use of oral contraceptives, previous breast biopsies and energy intake.

[4] *P* value for linear trend in tertiles following Benjamini & Hochberg procedure (based on 31 independent models).

[5] Geometric mean ± geometric SDs.

Figure 1. Difference in mammographic density percentage as a smooth of serum relative concentrations of the main fatty acid groups among premenopausal women in Madrid, Spain, 2013–2015, n=1196.

Curves represent adjusted mean differences (solid lines) and 95% confidence intervals (dashed lines) based on restricted quadratic splines for saturated, *cis*-monounsaturated, *trans*-monounsaturated, n-6 polyunsaturated, n-3 polyunsaturated fatty acids, and the log-transformed ratio of n-6 to n-3 polyunsaturated fatty acids with knots at their 5th, 50th, and 95th percentiles. The reference value for each type of fatty acid (mean difference = 0) was set at the median of the first tertile (49.2%, 7.75%, 1.16%, 27.1%, 2.44%, and 6.36, respectively). Mean differences were obtained from linear regression models adjusted for age, educational level, body mass index, waist circumference, parity, use of oral contraceptives, previous breast biopsies, and energy intake. Bars represent the histogram of each type of fatty acid.