Research article
Cytochrome P450 1A2 (CYP1A2) activity, mammographic density, and oxidative stress: a cross-sectional study
Chi-Chen Hong1, Bing-Kou Tang2, Venketeshwer Rao3, Sanjiv Agarwal3, Lisa Martin1, David Tritchler1, Martin Yaffe4 and Norman F Boyd1

1Division of Epidemiology and Statistics, Ontario Cancer Institute, Toronto, Ontario, Canada
2Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada
3Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada
4Medical Imaging Research Sunnybrook and Women’s College Health Sciences Centre, Toronto, Ontario, Canada

Corresponding author: Norman F Boyd, boyd@uhnres.utoronto.ca

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Abstract

Introduction Mammographically dense breast tissue is a strong predictor of breast cancer risk, and is influenced by both mitogens and mutagens. One enzyme that is able to affect both the mitogenic and mutagenic characteristics of estrogens is cytochrome P450 1A2 (CYP1A2), which is principally responsible for the metabolism of 17β-estradiol.

Methods In a cross-sectional study of 146 premenopausal and 149 postmenopausal women, we examined the relationships between CYP1A2 activity, malondialdehyde (MDA) levels, and mammographic density. In vivo CYP1A2 activity was assessed by measuring caffeine metabolites in urine. Levels of serum and urinary MDA, and MDA–deoxyguanosine adducts in DNA were measured. Mammograms were digitized and measured using a computer-assisted method.

Results CYP1A2 activity in postmenopausal women, but not in premenopausal women, was positively associated with mammographic density, suggesting that increased CYP1A2 activity after the menopause is a risk factor for breast cancer. In premenopausal women, but not in postmenopausal women, CYP1A2 activity was positively associated with serum and urinary MDA levels; there was also some evidence that CYP1A2 activity was more positively associated with percentage breast density when MDA levels were high, and more negatively associated with percentage breast density when MDA levels were low.

Conclusion These findings provide further evidence that variation in the activity level of enzymes involved in estrogen metabolism is related to levels of mammographic density and potentially to breast cancer risk.

Keywords: caffeine metabolic ratio, CYP1A2, malondialdehyde, malondialdehyde deoxyguanosine adducts, mammographic density

Introduction

Increased amounts of stromal and epithelial tissue in the breast is a strong independent risk factor for breast cancer, and they appear as light areas on a mammogram because they are radiologically dense. These areas are referred to as ‘mammographic density’. In contrast, fat appears dark on a mammogram because it is radiologically lucent [1].

Like breast cancer, epidemiologic evidence for breast density suggests that endocrine factors play a role [1]. Mammographic density levels decline with increasing age, onset of menopause, increasing body weight and parity, and younger age at first childbirth [1]. Density levels increase with hormone replacement therapy [2], and are reduced with tamoxifen [3] and a gonadotrophin release agonist [4].

In addition to mitogens, mammographic density may also be influenced by mutagens. Relationships between mammographic density and the mutagen malondialdehyde (MDA), an end product of lipid peroxidation, have been observed in two studies [5,6], suggesting that breast density levels are also affected by mutagenic events that can

AAMU = 5-acetylamino-6-amino-3-methyluracil; AFMU = 5-acetylamino-6-formylamino-3-methyluracil; BMI = body mass index; CMR = caffeine metabolic ratio; CYP1A2 = cytochrome P450 1A2; dG-MDA = malondialdehyde–deoxyguanosine (adducts); HPLC = high-performance liquid chromatography; MDA = malondialdehyde; 1U = 1-methylurate; 1, 7U = 1,7-dimethylurate; 1X = 1-methylxanthine; WHR = waist–hip ratio.
lead to DNA damage [7,8], and increased cell proliferation [9]. The main DNA adduct is malondialdehyde–deoxyguanosine (dG-MDA), and this and other MDA adducts are twofold to threefold greater in breast tissue from patients with breast cancer than in breast tissue from those without breast cancer [10]. Increased levels of circulating MDA have also been detected in breast cancer patients compared with noncancer patients [11].

Of human P450s that are capable of oxidizing estradiol, cytochrome P450 1A2 (CYP1A2) has the highest catalytic activity, resulting in the production of 2-hydroxy and 4-hydroxy metabolites. The absolute level of 2-hydroxylation of estrone, which is mediated by CYP1A2, is six to seven times greater than the extent of 4-hydroxylation mediated by this enzyme [12]. Although CYP1A2 is a hepatic enzyme that is not expressed in the breast, it can potentially affect circulating estrogen levels. Circulating sex hormone levels, in turn, have been shown in prospective studies of postmenopausal women to be associated with future risk for developing breast cancer [13]. Differences in CYP1A2 mRNA levels in human liver have also been reported, and it has been suggested that these differences in CYP1A2 expression may play a role in various cancers [14,15]. Figure 1 shows the estrogen metabolites resulting from CYP1A2 function and how these metabolites may potentially have an impact on breast density levels [16]. Of these metabolites, 4-hydroxyestrogens are known to be estrogenic and are thought to be carcinogenic. Most studies agree, however, that 2-hydroxyestrogens are not dangerous because the metabolite is nonestrogenic and has been determined to be non-tumorigenic in animal models [17]. Catecholestrogens can contribute to carcinogenesis by becoming a source of reactive oxygen species and entering into redox cycling (i.e. repeated cycles of oxidation and reduction between catechol and quinone estrogens). In particular, 4-hydroxyestrogens can become a source of potentially genotoxic and cytotoxic reactive oxygen species (ROS) that can bind to DNA to create depurinating adducts. ROS can also participate in lipid peroxidation to create the mutagen malondialdehyde (MDA), which is a risk factor for mammographic density. These mechanisms are postulated to be inhibitory (−) or stimulatory (+) for development of mammographic density.

The goals of the present cross-sectional study of premenopausal and postmenopausal women were to determine whether CYP1A2 activity is associated with mammographic density, whether CYP1A2 activity is related to MDA levels (serum, urinary, and dG-MDA), and whether MDA levels are associated with breast density.
Methods
The methods for this study are published in detail elsewhere [19] and are only briefly described here. Ethical approval for the study protocol was given by the Human Subjects Review Committee at the University of Toronto, Canada.

Source of study subjects
Between 1994 and 1997, potential participants were identified from the mammographic units of Mount Sinai, Women's College, and St. Michael's Hospital in Toronto. The extent of mammographic density for all patients was visually estimated by a radiologist and expressed as a percentage of breast area on a five-point scale. The number of patients recruited into each of the five categories of radiological density were as follows: <10%, n = 101; 10% to <25%, n = 62; 25% to <50%, n = 60; 50% to <75%, n = 60; and ≥75%, n = 99.

Recruitment
Potential participants were sent a letter and subsequently telephoned about the study. Premenopausal women were eligible if they were menstruating regularly, not pregnant or breast-feeding, and had not had a hysterectomy or oophorectomy. Postmenopausal women were eligible if they had spontaneous amenorrhea for at least 12 months, or had had a hysterectomy and were 50 years of age or older, or had had a bilateral oophorectomy at any age. A woman was excluded if she was taking any type of exogenous hormone preparation, had breast augmentation or reduction, or had a personal history of breast cancer or was being investigated for breast cancer. In total, 382 women agreed to participate in the study, representing 88% of those who were contacted and found to be eligible.

Measurements
Blood samples were collected after a 12-hour overnight fast, and during the luteal phase of the menstrual cycle (days 20–24) for premenopausal women. The mammogram closest to the time of the blood draw was used (mean difference 32 weeks). Twenty-four hour urine samples were also collected.

Obtaining consent for the phenotyping study
Because examination of CYP1A2 activity in relation to mammographic density was not a goal of the original study, patients were subsequently mailed a letter describing the goals of the phenotypic component of this study, and written consent was obtained to phenotype CYP1A2 activity. Information on ethnicity was also obtained at this time. Of 382 eligible women (193 premenopausal and 189 postmenopausal), 357 (93%) gave consent. Eight women could not be contacted because they had moved and could not be traced through either telephone directories or their physicians. Sixteen women were nonresponders after a minimum of four telephone reminders, and one did not provide consent.

Of the 357 women, 40 (11.2%; 29 premenopausal and 11 postmenopausal) did not have urine samples and 22 (6.2%; seven premenopausal and 15 postmenopausal) had insufficient excretion of caffeine metabolites for estimating CYP1A2 activity, leaving 146 postmenopausal and 149 postmenopausal women in whom CYP1A2 activity was measurable. This represented 93% of women with urine samples and 82% of eligible women who provided consent. The overall participation rate for this portion of the study was 77% (295/382).

Ethnicity
By questionnaire, each participant was asked their country of birth as well as the countries of birth for each of their parents and grandparents. They were also asked the question, 'What is your ethnic or cultural background?' and were given instructions to mark all appropriate categories. They were classified as follows: black; white (e.g. British, French, European, Latin/South American of European background); native/aboriginal people of North America (North American Indian, Inuit, Métis); East Asian (e.g. Chinese, Japanese, Korean, Vietnamese); South Asian (e.g. Indian from India, Pakistani, Punjabi, Tamil); other, with specification; and 'don't know'. Because of low numbers in groups other than Caucasians, the categories were collapsed and described as Caucasian (white), East Asians, Jewish, and other.

Epidemiologic and anthropometric data
Information about epidemiologic risk factors for breast density and breast cancer was collected by questionnaire, and dietary information was obtained using a list-based food frequency questionnaire developed by Block and coworkers [20]. Each woman was weighed and measured for height, and waist and hip circumference.

CYP1A2 activity
Caffeine (1, 3, 7-trimethylxanthine) is metabolized by CYP1A2 and has been used to evaluate CYP1A2 activity in vivo [21]. The best urinary metabolic ratio appears to be [5-acetylamino-6-formylamino-3-methyluracil (AFMU) + 1-methylurate (1U) + 1-methylxanthine (1X)]/1,7-dimethylurate (1, 7U), which is referred to as the caffeine metabolic ratio (CMR) [22]. The CMR is not dependent on renal flow [23], and is based only on metabolic end products of caffeine metabolism, thus making the amount and timing of urine collection relatively unimportant [24]. Measurement of the CMR in 24-hour urine samples has been shown to be a viable method for assessing CYP1A2 intake in populations with widespread caffeine use [25]. In the present study, 93% of women consumed sufficient levels of caffeine to estimate CMR. Because daily caffeine intake is relatively...
constant, urinary recovery of caffeine metabolites over a 24-hour period reflects CMRs observed with standardized dosing [25].

Urinary caffeine metabolites were measured by HPLC as previously described [21], except for a modification of the composition of the mobile phase. The mobile phase was composed of 1.3% isopropanol, 0.2% isonitrile, and 0.1% phosphoric acid. The caffeine metabolites were eluted at 1 ml/min and detected by ultraviolet absorbance (0.05) at 280 nm. The retention times of 1U, 1X and 1, 7U and the internal standard (N-acetyl-p-aminophenol) were 9.9, 11.9, 29.8, and 14.2 min, respectively.

Urinary AFMU was first deformylated to stable 5-acetylamino-6-amino-3-methyluracil (AAMU) and then measured using the HPLC method reported by Tang and coworkers [21]. The mobile phase consisted of 0.075% acetic acid and 0.075% phosphoric acid. AAMU and the internal standard (hydrobenzyl alcohol) were eluted at a flow of 0.9 ml/min and monitored by ultraviolet absorbance (0.04) at 263 nm. The retention times of AAMU and the internal standard were 13 and 36 min, respectively.

A standard urine sample with known caffeine metabolite concentrations was analyzed across all days of sample analyses with an interassay coefficient of variation of 9%. Accuracy of the CYP1A2 measurement did not vary with caffeine intake. After adjustment for smoking status, age, body mass index (BMI), waist–hip ratio (WHR), and ethnic–caffeine intake. After adjustment for smoking status, age, body mass index (BMI), waist–hip ratio (WHR), and ethnic–

Serum antioxidants
Serum levels of α-tocopherol, retinol, and five commonly reported carotenoids (α- and β-carotene, lutein, lycopene and cryptoxanthin) were extracted using hexane and analyzed by reverse phase HPLC, C18 column, and absorbance detector. Peaks were identified and quantified by using their respective standards [27].

Serum MDA, urinary MDA, dG-MDA adducts
MDA in serum and urine was measured in triplicate by HPLC determination of thiobarbituric acid derivatives, as described by Bird and coworkers [28]. The sample was extracted with trichloroacetic acid and then heated with thiobarbituric acid. The thiobarbituric acid–MDA complex was separated using HPLC and the absorbance measured at 535 nm. Total urinary MDA level excreted over a 24-hour period was calculated as concentration of urinary MDA multiplied by total urinary volume collected. Study results with total urinary MDA were similar to those obtained when total urinary MDA levels were expressed per millimole creatinine. Results shown here are those obtained with total urinary MDA levels unadjusted for creatinine.

Precipitated DNA was treated with RNase A and RNase T1 to remove RNA contamination. Isolated DNA was hydrolyzed using nuclease P1 and alkaline phosphatase, and the amount of dG-MDA adducts were determined by reverse-phase HPLC, C18 column, and fluorescence detection (340 nm excitation, 518 nm emission) using 30% as the mobile phase, as previously described [29].

Serum estradiol
Serum estradiol levels in premenopausal women were measured at the London Regional Cancer Center in Ontario, Canada in the laboratory of Geoffrey Hammond [30]. Serum estradiol levels in postmenopausal women were measured by Esoterix Center for Clinical Trials (Calabasas Hills, California, USA) [19].

Mammographic density
Breast density measurements were made using a randomly selected, craniocaudal (viewing from above, down) mammographic view of one breast from each woman. Mammograms were digitized using a Luminys model 85 (Eastman Kodak Company, Rochester, NY, USA) and randomly ordered for analysis. The observer (NFB) selected a threshold gray value to separate the image of the breast from the background. A second threshold was selected to identify the edges of regions representative of radiographically dense tissue. Summation of pixels within these areas gave a measure of the area of density in the breast. The percentage of radiographic density is the area of dense tissue divided by the entire projected area of the breast multiplied by 100. Further details of this method are given elsewhere [31].

Statistical methods
Data analyses were carried out using the SAS statistical software package (version 6.12; SAS Institute Inc., Cary, NC, USA) [32]. Data were inspected for normality and, when necessary, transformed to approximate a normal distribution. This included square root transformation of percentage breast density, and natural log transformation of CYP1A2 activity, BMI, and MDA levels. Because of differences in results between premenopausal and postmenopausal women, all data are presented stratified by menopausal status.

Partial correlations were used to control for potential confounders when relationships between serum, urinary, and dG-MDA levels were assessed.
Relationships between CYP1A2 phenotype, MDA levels, and breast density were assessed using multiple regression analysis. For descriptive purposes, relationships across quartiles were also provided with a test for trend. Although these results are based on analyses of transformed data, they are presented in their original units with 95% confidence intervals. Age, BMI, WHR, ethnicity, and smoking status were included in all models unless indicated. Age and body size were included as potential confounders because increased oxidant status and lipid peroxidation is related to advancing age, central obesity, hyperinsulinemia, insulin resistance, dyslipidemia, and blood glucose [33-36]. In the present study, serum MDA levels were positively associated with BMI measurements in both premenopausal and postmenopausal women (P ≤ 0.005). Age and body size are also important determinants of breast density levels [1]. Adjustments were made for smoking status because tobacco smoke is inversely associated with mammographic density [37] and is a known inducer of CYP1A2 activity [38]. Associations between CYP1A2 and MDA levels, as well as associations between MDA levels and mammographic density, were further adjusted for serum antioxidant levels.

To illustrate interactions between CYP1A2 activity and MDA levels (i.e. two continuous variables) on percentage density, relationships (simple slopes) between CYP1A2 activity and percentage density were determined for (conditional on) three levels of oxidative stress: at mean level, at one standard deviation above the mean, and one standard deviation below the mean [39].

Of 295 women with CYP1A2 results, 251 (85%) were non-Jewish Caucasians, 10 were East Asians, 15 were Jewish, and 19 were from other ethnic groups. To assess the possibility that ethnicity may be a potential confounder of findings, all analyses were performed on the 295 women with CYP1A2 results, with and without adjustment for ethnicity. All analyses were then repeated with only the 251 non-Jewish Caucasians. Findings from all three sets of analyses were similar, suggesting that ethnicity was not a strong confounder in the study. Results shown in this report are those performed on the 295 women with adjustment for ethnicity.

Results

Study subject characteristics

There were 146 premenopausal and 149 postmenopausal women with CYP1A2 phenotype data, for whom selected characteristics are shown in Table 1. Of the participants 85% were Caucasian, 3.4% were East Asian, 5.1% were Jewish, and 6.4% were from other ethnic groups. The mean age was 45 years in premenopausal and 56 years in postmenopausal women. The two groups were similar in age at menarche, age at first birth, and total number of livebirths.

Postmenopausal women were slightly heavier, with higher average BMI and WHR. They also had lower levels of percentage mammographic density, and higher serum and urinary MDA levels.

Compared with Caucasians, premenopausal and postmenopausal East Asian women and premenopausal Jewish women were lighter, with lower mean BMIs and greater levels of percentage breast density. Among premenopausal women, East Asians had higher levels of urinary MDA than did Caucasians. In postmenopausal women, compared with Caucasians, East Asians were older at age of first birth, and had higher levels of CYP1A2 activity and urinary MDA. Jewish women had lower CYP1A2 activity than did Caucasians.

In premenopausal women, those without CYP1A2 data had higher urinary MDA levels than did those with phenotype data. In postmenopausal women, those without CYP1A2 results weighed less, and had lower mean BMI and urinary MDA levels. Percentage density was on average 14% higher in these patients than in women with CYP1A2 results.

CYP1A2 activity

The CMR in premenopausal women ranged from 0.8 to 23.5, with a mean ± standard deviation of 6.2 ± 2.9 and a median of 5.8. In postmenopausal women the ratio ranged from 1.8 to 17.1, with a mean ± standard deviation of 5.9 ± 2.3, and median of 5.4. This magnitude of interindividual variation is similar to those in previous reports showing up to a 30-fold variation in enzyme activity [40,41]). The population distribution of CMR in the present study was log-normal, which is similar to findings of other studies using the same urinary ratio [25,38,40,41].

MDA measures

Postmenopausal women had higher serum (F = 18.08, P < 0.0001) and urinary (F = 24.05, P < 0.0001) MDA levels than did premenopausal women, but dG-MDA levels were not different between the two groups (P = 0.41). These findings were not altered when further adjusted for age, BMI, and WHR. The coefficient of variation associated with each MDA measure (untransformed values) was higher in premenopausal than in postmenopausal women (41.4 versus 37.0 for serum MDA; 52.1 versus 44.6 for urinary MDA; and 76.2 versus 56.0 for dG-MDA).

In premenopausal women, serum and urinary MDA levels were weakly correlated before (r = 0.15, P = 0.08) and after (r = 0.21, P = 0.02) adjustment for age, BMI, and WHR. Neither measure, however, was correlated with dG-MDA levels (P > 0.28). In postmenopausal women, serum MDA levels were positively associated with urinary (r = 0.17, P = 0.03) and dG-MDA (r = 0.17, P = 0.04) levels.

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Table 1

Selected characteristics of study subjects by ethnicity and menopausal status

|                     | With CYP1A2 results | No CYP1A2 results |
|---------------------|---------------------|-------------------|
|                     | Caucasian:          | East Asian:       |
|                     | pre (n = 125):      | pre (n = 4):      |
|                     | post (n = 126)      | post (n = 6)      |
|                     | Jewish:             | Other:            |
|                     | pre (n = 10):       | pre (n = 7):      |
|                     | post (n = 5)        | post (n = 12)     |
|                     | Other:              | Pre (n = 36):     |
|                     | pre (n = 7):        | post (n = 26)     |

|                  |                  |                  |
|------------------|------------------|------------------|
| **Premenopausal**| **Risk factors** |                  |
| Age (years)      | 44.8 (4.8)       | 46.8 (4.5)       |
| Weight (kg)      | 68.1 (16.6)      | 54.8 (3.1)       |
| BMI (kg/m²)      | 25.3 (6.1)       | 23.2 (0.9)       |
| WHR              | 0.75 (0.06)      | 0.75 (0.04)      |
| Age at menarche  | 12.8 (1.5)       | 11.5 (1.3)       |
| Age at first birth (years) & 27.8 (5.7) & 29.5 (0.7) & 29.5 (2.7) & 25.6 (9.7) & 29.0 (6.4) |
| Number of live births | 1.4 (1.2) | 1.0 (1.2) | 2.1 (1.3) | 1.6 (1.3) | 1.5 (1.2) |
| Mammographic density (%)⁶ | 27.4 (23.2) | 47.6 (17.0) | 40.2 (22.7) | 25.9 (20.0) | 28.4 (20.5) |
| CYP1A2 activity⁷ | 6.31 (2.95) | 6.52 (0.98) | 4.98 (1.04) | 6.11 (3.50) | - |

|                  |                  |                  |
| **Markers of oxidative stress** |                  |                  |
| Serum MDA (µmol/l) | 1.47 (0.58) | 1.52 (0.47) | 1.53 (1.05) | 1.77 (0.68) | 1.45 (0.66) |
| Urinary MDA (µmol/day) | 2855.3 (1514.1) | 3591.6 (1365.9) | 2813.0 (1392.2) | 2293.9 (1116.9) | 3729.4 (1365.9) |
| dG-MDA (µmol/mg DNA) | 200.8 (157.9) | 232.8 (115.7) | 221.4 (164.6) | 169.3 (76.27) | 180.2 (135) |

|                  |                  |                  |
| **Postmenopausal**| **Risk factors** |                  |
| Age (years)      | 56.3 (4.6)       | 54.8 (3.8)       |
| Weight (kg)      | 72.5 (16.6)      | 55.8 (9.8)       |
| BMI (kg/m²)      | 26.8 (6.2)       | 23.2 (4.7)       |
| WHR              | 0.77 (0.07)      | 0.77 (0.08)      |
| Age at menarche  | 13.1 (1.6)       | 13.0 (1.7)       |
| Age at menopause | 49.1 (4.8)       | 50.8 (3.3)       |
| Age at first birth (years) & 26.0 (5.2) | 34.6 (6.8) | 22.8 (3.8) | 31.0 (8.7) | 25.4 (4.1) |
| Number of live births | 1.8 (1.5) | 1.8 (1.5) | 2.2 (1.6) | 1.0 (1.0) | 1.4 (1.4) |
| Mammographic density (%)⁶ | 19.8 (18.5) | 48.2 (8.9) | 22.3 (24.6) | 30.1 (23.8) | 36.0 (22.1) |
| CYP1A2 activity⁷ | 5.73 (2.18) | 8.86 (1.83) | 3.49 (1.75) | 6.64 (2.81) | - |

|                  |                  |                  |
| **Markers of oxidative stress** |                  |                  |
| Serum MDA (µmol/l) | 1.75 (0.65) | 1.83 (0.59) | 1.69 (0.74) | 1.64 (0.7) | 1.84 (0.72) |
| Urinary MDA (µmol/day) | 3518.5 (1587.5) | 4636.2 (1348.3) | 3412.9 (978.9) | 3717.0 (1926.2) | 2449.3 (1301.8) |
| dG-MDA (µmol/mg DNA) | 197.7 (112.6) | 149.9 (107.2) | 158.2 (108.1) | 212.9 (80.8) | 203.2 (109.6) |

Values are expressed as mean (standard deviation). ¹Premenopausal (pre): n = 86 for age at first birth, n = 120 for urinary malondialdehyde (MDA), and n = 121 for malondialdehyde–deoxyguanosine (dG-MDA). ²Postmenopausal (post): n = 117 for menopausal age, n = 97 for age at first birth, and n = 123 for dG-MDA. ³Pre: n = 2 for age at first birth. Post: n = 5 for age at first birth. ⁴Pre: n = 8 for age at first birth and n = 9 for dG-MDA. Post: n = 4 for age at first birth and dG-MDA. ⁵Pre: n = 5 for age at first birth. Post: n = 11 for menopausal age and n = 8 for age at first birth. ⁶Pre: n = 25 for age at first birth, n = 7 for urinary MDA, and n = 24 for dG-MDA. Post: n = 23 for age at menopause, n = 14 for age at first birth, n = 15 for urinary MDA, and n = 25 for dG-MDA. ⁷Proportion of breast area occupied by dense tissue. ⁸Estimated by caffeine metabolic ratio (see text for details). BMI, body mass index; CYP1A2, cytochrome P450 1A2; WHR, waist–hip ratio.
After adjustment for age, BMI, and WHR, serum MDA continued to be associated with dG-MDA ($r = 0.19, P = 0.02$), but it was only weakly associated with urinary MDA levels ($r = 0.14, P = 0.09$). Urinary and dG-MDA levels were not correlated ($P \geq 0.13$).

**CYP1A2 activity and mammographic density**

Relationships between CYP1A2 activity and percentage mammographic density levels are shown in Table 2. Results were initially adjusted for age, ethnicity, and smoking. Findings were then further adjusted for total estrogen levels to determine whether CYP1A2 function was independently associated with breast density levels without the influence of estrogen. Finally, findings were further adjusted for age at menarche, age at menopause (in postmenopausal women only), number of live births, BMI, WHR, family history of breast cancer, and smoking status.

CYP1A2, as an indicator of CYP1A2 activity, was not related to percentage density in premenopausal women after adjustment for age, ethnicity, and smoking ($P = 0.49$). In contrast, CYP1A2 in postmenopausal women was positively associated with premenopausal women after adjustment for age, ethnicity, and smoking ($\beta = 1.57 \pm 0.60, F = 6.89, P = 0.01$) as well as total estrogen ($\beta = 1.30 \pm 0.64, F = 4.05, P = 0.05$). The relationship was attenuated and no longer significant when further adjusted for other risk factors for breast density ($\beta = 0.94 \pm 0.64, F = 2.36, P = 0.13$), although the $P$ trend remained significant across quartiles of CYP1A2 ($P = 0.05$). The positive association between CYP1A2 and breast density was primarily due to a lower mean percentage density level in the first quartile of CYP1A2.

**CYP1A2 activity and malondialdehyde levels**

Relationships between CYP1A2 activity and MDA levels are shown in Table 3. CYP1A2 activity was positively associated with serum ($P = 0.03$) and urinary ($P = 0.04$) MDA levels in premenopausal but not in postmenopausal women ($P \geq 0.58$). These relationships in premenopausal women were not altered when further adjusted for serum levels of the antioxidants tocopherol, retinol, β-carotene, β-cryptoxanthin, lycopene, and lutein (serum MDA: $P = 0.04$; urinary MDA: $P = 0.04$). No relationships were observed between CYP1A2 activity and dG-MDA levels in either menopausal group before ($P \geq 0.34$) or after adjustment ($P \geq 0.32$) for serum antioxidant levels.

**MDA levels and mammographic density**

Relationships between MDA levels and percentage breast density are shown in Table 4. No associations were observed between serum, urinary, or dG-MDA levels and percentage breast density in either premenopausal or postmenopausal women after adjustment for age, ethnicity, BMI, WHR, and smoking status. Further adjustments for serum antioxidant levels did not alter these findings. However, in premenopausal and postmenopausal women combined, we observed a positive association between urinary MDA levels and percentage mammographic density when adjusted for age, ethnicity, weight (instead of BMI), WHR, smoking status, and menopausal status ($\beta = 0.47 \pm 0.22, F = 4.45, P = 0.04$). With further adjustment for serum antioxidant levels, however, the relationship was no longer sig-

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**Table 2**

| Model adjustments | CMR: $\beta$ (SE) $^1$ | F | \(P\) | Q1 | Q2 | Q3 | Q4 | $P$ for trend |
|-------------------|------------------------|---|-----|----|----|----|----|--------------|
| **Premenopausal (n = 146)$^3$** | | | | | | | | |
| Age, ethnicity, smoking$^2$ | 0.29 (0.52) | 0.31 | 0.58 | 26.7 (14.5–42.6) | 28.4 (16.9–42.8) | 30.7 (19.0–45.2) | 29.9 (17.9–45.0) | 0.57 |
| + total estrogen | 0.36 (0.52) | 0.48 | 0.49 | 26.1 (13.8–42.3) | 28.0 (16.5–42.5) | 30.2 (18.4–44.8) | 30.3 (18.0–45.8) | 0.49 |
| + other covariates | -0.16 (0.38) | 0.17 | 0.68 | 20.1 (10.3–33.1) | 20.9 (11.7–32.8) | 21.2 (12.4–32.2) | 18.5 (10.0–29.6) | 0.72 |
| **Postmenopausal (n = 149)$^4$** | | | | | | | | |
| Age, ethnicity, smoking | 1.57 (0.60) | 6.89 | 0.01 | 14.1 (6.6–24.3) | 30.2 (18.4–45.1) | 24.8 (14.7–37.5) | 31.0 (20.9–43.2) | 0.01 |
| + total estrogen | 1.30 (0.64) | 4.05 | 0.05 | 12.8 (5.6–22.9) | 27.2 (15.7–41.7) | 22.5 (12.9–34.8) | 27.4 (17.6–39.4) | 0.03 |
| + other covariates | 0.94 (0.61) | 2.36 | 0.13 | 14.6 (5.7–27.6) | 25.0 (13.0–41.0) | 19.0 (8.6–33.5) | 28.2 (15.8–44.1) | 0.05 |

$^1$ Associated with log transformed caffeine metabolic ratio (CMR) as the independent variable and square-root transformed percentage breast density as the dependent variable. $^2$ Statistical models are initially adjusted for age, ethnicity, and smoking status. Models are subsequently adjusted for total estrogen, and then for other covariates, which include adjustments for age at menarche, age at menopause (postmenopausal only), number of live births, body mass index (BMI), waist–hip ratio (WHR), and family history of breast cancer. $^3$ n = 144 for estrogen adjusted analysis and n = 143 for covariate adjusted analysis. $^4$ n = 127 for estrogen adjusted analysis and n = 118 for covariate adjusted analysis.
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Table 3

CYP1A2 activity and malondialdehyde levels

|                    | Quartiles of CMR: mean MDA (95% confidence interval) |
|--------------------|------------------------------------------------------|
|                    | Q1                                      Q2                                      Q3                                      Q4                                      |
|                    | F    | P   | 1.37 (1.15–1.63) | 1.52 (1.30–1.77) | 1.50 (1.28–1.74) | 1.62 (1.38–1.91) | 0.06 |
| Serum MDA (µmol/l) | 1    | 0.15 (0.07) | 1.33 (1.12–1.57) | 1.39 (1.19–1.62) | 1.47 (1.27–1.71) | 1.53 (1.31–1.79) | 0.05 |
| Serum MDA adjusted for antioxidants (µmol/l) | 2    | 0.14 (0.07) | 2.35 (1.81–3.06) | 2.44 (1.92–3.09) | 3.06 (2.44–3.84) | 2.78 (2.18–3.53) | 0.06 |
| Total urinary MDA (µmol/day) | 1    | 0.23 (0.11) | 1.81 (1.30–3.09) | 2.90 (2.29–3.68) | 2.75 (2.14–3.54) | 0.08 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | 3    | 0.24 (0.11) | 1.81 (1.30–3.09) | 2.90 (2.29–3.68) | 2.75 (2.14–3.54) | 0.08 |
| dG-MDA (p mol/mg DNA) | 1    | -0.13 (0.15) | 186.8 (126.5–275.9) | 160.1 (112.5–227.6) | 157.0 (111.6–220.8) | 153.6 (106.9–220.6) | 0.29 |
| dG-MDA adjusted for antioxidants (p mol/mg DNA) | 4    | -0.09 (0.16) | 187.2 (126.0–278.1) | 164.7 (115.0–235.7) | 143.9 (101.7–203.6) | 163.0 (113.0–235.2) | 0.35 |
| Postmenopausal (n = 149) | 4    | -0.00 (0.09) | 1.73 (1.45–2.07) | 1.63 (1.36–1.95) | 1.55 (1.31–1.83) | 1.72 (1.49–1.99) | 0.86 |
| Serum MDA (µmol/l) | 1    | 0.08 (0.09) | 1.71 (1.42–2.04) | 1.66 (1.38–1.99) | 1.62 (1.35–1.94) | 1.81 (1.55–2.10) | 0.52 |
| Serum MDA adjusted for antioxidants (µmol/l) | 2    | -0.06 (0.11) | 4.06 (3.24–5.09) | 3.75 (2.97–4.72) | 3.77 (3.05–4.65) | 3.69 (3.62–3.76) | 0.43 |
| Total urinary MDA (µmol/day) | 1    | -0.12 (0.11) | 4.02 (3.20–5.04) | 3.82 (3.03–4.80) | 4.08 (3.25–5.11) | 3.70 (3.06–4.47) | 0.20 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | 3    | -0.12 (0.11) | 4.02 (3.20–5.04) | 3.82 (3.03–4.80) | 4.08 (3.25–5.11) | 3.70 (3.06–4.47) | 0.20 |
| dG-MDA (p mol/mg DNA) | 1    | 0.16 (0.16) | 141.2 (101.4–196.6) | 135.1 (96.6–188.9) | 133.9 (98.2–182.5) | 156.8 (119.4–205.9) | 0.57 |
| dG-MDA adjusted for antioxidants (p mol/mg DNA) | 4    | 0.17 (0.17) | 153.7 (109.3–216.2) | 143.9 (101.9–203.2) | 147.1 (105.2–205.6) | 167.0 (125.4–222.3) | 0.56 |

1Log transformed. All statistical models are adjusted for age, ethnicity, body mass index (BMI), waist–hip ratio (WHR), and smoking status. 2Models are further adjusted for serum levels of the antioxidants tocopherol, retinal, β-carotene, β-cryptoxanthin, lycopena, and lutein. 3n = 143 for serum malondialdehyde (MDA) with adjustment for antioxidants; n = 141 for urinary MDA and n = 138 with adjustment for antioxidants; and n = 141 for malondialdehyde–deoxyguanosine (dG-MDA) adducts and n = 139 with adjustment for antioxidants. 4n = 145 for dG-MDA. CMR, caffeine metabolic ratio.

significant (β = 0.35 ± 0.23, F = 2.29, P = 0.13). The relationship was also attenuated slightly when height was further added to the model (β = 0.35 ± 0.22, F = 2.62, P = 0.11). If all women who participated in the original study were included, including those without CYP1A2 results, then the positive association observed between urinary MDA levels and breast density after adjustment for age, weight, smoking status, WHR, and menopausal status was significant (n = 335; β = 0.53 ± 0.22, F = 5.67, P = 0.02), even after adjustment for height (β = 0.44 ± 0.22, F = 3.97, P = 0.05), but not with further adjustment for serum antioxidant levels (β = 0.31 ± 0.23, F = 1.85, P = 0.18). Similarly, among all women there was a positive association between urinary MDA and breast density if adjustment was for waist circumference (the anthropometric variable most closely associated with breast density levels), smoking status, height, and menopausal status (β = 0.44 ± 0.22, F = 4.06, P = 0.04). The relationship was attenuated by further adjustment for serum antioxidant levels (β = 0.32 ± 0.22, F = 2.10, P = 0.15).

Interaction between CYP1A2 activity and MDA levels
Although not originally hypothesized, we tested for possible interactions between CYP1A2 function and MDA levels with respect to mammographic density. Relationships between CYP1A2 activity and mammographic density according to MDA levels are shown in Fig. 2. Although not statistically significant, interactions in premenopausal
The interaction between dG-MDA adducts and CYP1A2 activity was statistically significant if an adjustment was made for weight instead of BMI (F = 4.67, P = 0.03). This interaction was slightly attenuated if a further adjustment was made for height (F = 3.36, P = 0.07). For both MDA measures, the associations between CYP1A2 activity and breast density were more negative at low levels of MDA, and became more positive as MDA levels increased. At low dG-MDA adduct levels (illustrated at 1 standard deviation), for example, there was a negative nonsignificant relationship between CYP1A2 activity and percentage breast density (β = -0.77 ± 0.50, F = 2.36, P = 0.13). At mean MDA level CYP1A2 activity was not associated with breast density (β = -0.03 ± 0.40, F = 0.00, P = 0.95), and at high levels of dG-MDA (illustrated at +1 standard deviation) there was a positive nonsignificant association between CYP1A2 activity and breast density (β = 0.72 ± 0.66, F = 1.16, P = 0.28). A similar but nonsignificant (P = 0.29) pattern of interaction between CYP1A2 function and serum MDA levels were observed for postmenopausal women, although in the instance of urinary MDA the pattern was reversed. Those with lower MDA levels showed more positive associations between CYP1A2 function and breast density levels (P = 0.35).

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Table 4

Malondialdehyde levels and mammographic density

| Quartiles of MDA: mean percentage density (95% confidence interval) | MDA1 (SE) | F | P | Q1 | Q2 | Q3 | Q4 | P for trend |
|---|---|---|---|---|---|---|---|---|
| Premenopausal (n = 146)3 | | | | | | | | |
| Serum MDA (µmol/l) | 0.57 (0.46) | 1.52 | 0.22 | 26.1 (17.7–36.1) | 25.7 (17.0–36.1) | 27.2 (18.9–36.9) | 26.3 (18.1–36.0) | 0.89 |
| Serum MDA adjusted for antioxidants (µmol/l)2 | 0.12 (0.51) | 0.06 | 0.81 | 28.4 (19.3–39.1) | 25.8 (17.0–36.5) | 23.6 (15.7–33.0) | 22.9 (15.0–32.6) | 0.26 |
| Total urinary MDA (µmol/day) | 0.24 (0.32) | 0.60 | 0.44 | 23.5 (15.5–33.2) | 27.9 (18.5–39.2) | 25.3 (17.6–34.4) | 26.9 (18.5–36.8) | 0.61 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | 0.17 (0.33) | 0.27 | 0.61 | 21.9 (14.0–31.5) | 26.5 (17.2–37.9) | 25.3 (17.4–34.7) | 24.3 (16.1–34.3) | 0.70 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.02 (0.22) | 0.01 | 0.94 | 18.8 (12.1–27.0) | 32.7 (22.8–44.4) | 31.5 (22.4–42.0) | 23.0 (15.7–31.8) | 0.91 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | 0.10 (0.23) | 0.18 | 0.67 | 19.8 (12.6–28.5) | 32.8 (22.4–45.1) | 28.2 (19.4–38.7) | 23.6 (15.9–32.8) | 0.58 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.33 (0.49) | 0.47 | 0.49 | 21.3 (13.4–31.1) | 24.7 (16.5–34.5) | 22.4 (14.1–32.7) | 21.2 (13.6–30.4) | 0.84 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.37 (0.52) | 0.52 | 0.47 | 22.3 (13.2–33.9) | 25.4 (16.8–35.7) | 23.4 (14.4–34.5) | 21.8 (13.7–31.9) | 0.77 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | 0.19 (0.39) | 0.24 | 0.63 | 25.1 (16.3–35.8) | 19.1 (11.5–28.7) | 29.5 (20.5–42.0) | 24.5 (11.6–34.0) | 1.00 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.04 (0.41) | 0.01 | 0.93 | 27.6 (17.9–39.6) | 19.8 (11.5–30.3) | 21.4 (13.5–31.1) | 24.7 (16.4–34.7) | 0.65 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.05 (0.28) | 0.04 | 0.85 | 22.6 (14.9–32.1) | 22.8 (14.1–33.5) | 24.2 (15.2–35.2) | 23.3 (14.8–33.7) | 0.86 |
| Total urinary MDA adjusted for antioxidants (µmol/day) | -0.11 (0.29) | 0.14 | 0.71 | 23.2 (14.7–33.6) | 23.5 (14.4–34.8) | 25.8 (16.1–37.8) | 21.9 (13.5–32.4) | 0.87 |

1β associated with log transformed malondialdehyde (MDA) levels as the independent variable and square-root transformed percentage breast density as the dependent variable. All statistical models are adjusted for age, ethnicity, body mass index, waist–hip ratio, and smoking status.
2Models are further adjusted for serum levels of the antioxidants tocopherol, retinal, β-carotene, β-cryptoxanthin, lycopene, and lutein. 3n = 141 for serum MDA with adjustment for antioxidants; n = 141 for urinary MDA and n = 138 with adjustment for antioxidants; and n = 141 for malondialdehyde–deoxyguanosine (dG-MDA) adducts and n = 139 with adjustment for antioxidants. 4n = 145 for dG-MDA.

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Figure 2

(a) Serum MDA

F = 0.00, P = 0.96

(b) Urinary MDA

F = 2.14, P = 0.15

(c) dG-MDA

F = 2.97, P = 0.09

Premenopausal

Postmenopausal

MDA Levels —— +1 SD ———— Mean ————-1 SD

Relationships between cytochrome P450 1A2 (CYP1A2) activity and percentage breast density according to (a) serum and (b) urinary malondialdehyde (MDA) levels, and (c) malondialdehyde–deoxyguanosine (dG-MDA) levels. All analyses are adjusted for age, ethnicity, body mass index (BMI), waist–hip ratio (WHR), and smoking status. Confounders were set at mean values and determined for Caucasian nonsmokers to illustrate relationships between CYP1A2 activity and percentage density according to MDA levels. Number of women in each group: premenopausal, n = 146 for serum MDA and n = 141 for urinary MDA and dG-MDA; and postmenopausal, n = 149 for serum and urinary MDA and n = 145 for dG-MDA. CMR, caffeine metabolic ratio.
Discussion
In premenopausal women, CYP1A2 activity, as estimated by the CMR, was not associated with breast density levels but was positively related to serum and urinary MDA levels. In postmenopausal women, higher CYP1A2 activity was consistently associated with higher levels of breast density, suggesting that increased CYP1A2 activity in this group is a risk factor for breast cancer. No associations, however, were observed between CYP1A2 activity and MDA levels.

In postmenopausal women, relationships were consistently positive between CYP1A2 activity and breast density levels. These observations are consistent with the findings of Riza and coworkers [42], who found that postmenopausal women with dense parenchymal breast patterns had 58% higher levels of 2-hydroxyestrone than did women with no density. Prospectively, however, both a higher [43] and a lower [44] ratio of 2-hydroxyestrone to 16α-hydroxyestrone have been associated with increased risk for breast cancer in postmenopausal women.

The positive relationship between CYP1A2 function and breast density levels in postmenopausal women was slightly attenuated by adjustment for total estradiol levels, but it remained statistically significant. This finding suggests that the relationship between CYP1A2 function and breast density is partially mediated by circulating estradiol levels, but that other factors are also important, such as catecholamines and other products of CYP1A2 activity. CYP1A2 is known to activate several heterocyclic and aromatic amine promutagens [15, 45, 46] as well as tobacco smoke-specific nitrosamines [47]. The enzyme is also inductive, and previous studies have shown variation in activity by sex, race, age, smoking status, body mass index, coffee and alcohol consumption, and exposure to various combustion products and contaminants [15, 38, 48-52].

Although CYP1A2 activity was not associated with breast density levels in premenopausal women, there was some evidence that the relationship may be modulated by oxidative burden. CYP1A2 activity was positively associated with breast density when urinary and dG-MDA levels were high and negatively associated when MDA levels were low. These relationships, however, did not reach statistical significance because of small sample size and the choice of confounders included in the statistical model. The interaction between dG-MDA adduct levels and CYP1A2 activity on breast density levels, for instance, was statistically significant when adjusted for weight instead of BMI.

An interaction between CYP1A2 activity and oxidative stress is biologically plausible because CYP1A2 metabolism of endogenous and exogenous substrates produce reactive oxygenated metabolites [53]. In premenopausal but not in postmenopausal women, we observed positive associations between CYP1A2 activity and serum and urinary MDA levels, as measures of lipid peroxidation. CYP1A2 function has also been positively associated with urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine which is a biomarker of oxidative DNA damage [54-56].

In premenopausal women, the observed interaction between CYP1A2 activity and oxidative burden may result from competing consequences. Against a background of low oxidative burden, increased CYP1A2 function may reflect the advantages of estrogen inactivation [17], with attendant increases in reactive oxygen species being inactivated by protective antioxidant systems. Against a background of high oxidative stress, however, the disadvantages associated with further increases in pro-oxidant levels may outweigh the benefits of increased estradiol inactivation.

An association between urinary MDA levels and mammographic density has been reported in two separate studies [5, 6]. We, however, did not replicate the observation when premenopausal and postmenopausal women were considered separately [5]. This was due to our choice of confounders and to our limited sample size. A statistically significant association was observed when premenopausal and postmenopausal women were combined and results were adjusted for weight or waist circumference instead of BMI. The association was slightly attenuated by further adjustment for height as a potential confounder and remained significant if sample size was maximized to include all women in the original study. Further adjustments for serum antioxidant levels, however, attenuated these relationships.

The weak and null associations observed between MDA measures and breast density may be partly due to confounding by correlates of body size. Increased pro-oxidant activity is related to central obesity, hyperinsulinemia, insulin resistance, dyslipidemia, and blood glucose levels [33-36]. In this study, both serum and urinary MDA levels were positively associated with BMI and weight. BMI and WHR, however, are inversely associated with breast density levels [1]. Thus, MDA and mammographic density may seem inversely related if not properly adjusted for body size and its correlates. In our study, serum MDA levels after adjustment for age and ethnicity was inversely associated with mammographic density levels in both premenopausal (β = -1.07 ± 0.61, F = 3.08, P = 0.08) and postmenopausal (β = -1.29 ± 0.58, F = 5.02, P = 0.03) women. With further adjustments for body size (BMI and WHR) and smoking status, the relationship between MDA levels and breast density became more positive in both premenopausal (β = 0.57 ± 0.46, F = 1.52, P = 0.22) and postmenopausal (β = -0.33 ± 0.49, F = 0.47, P = 0.49) women, although neither was statistically significant.
Weak correlations between various MDA measurements may in part explain why these measures show different findings. This is consistent with previous observations that markers of oxidative DNA damage and lipid peroxidation may not be strongly correlated, and suggests that no single measure can be used as an indicator of in vivo oxidative stress [57]. MDA levels are determined by a number of factors apart from endogenous lipid peroxidation. Exogenous dietary intake, for instance, is estimated to represent 10–18% [58] and as high as 50–60% [59] of urinary MDA levels [60,61], and it is difficult to adjust for in data analysis because the MDA content of foods varies widely and is affected by lipid type, storage, and cooking methods. Regarding dG-MDA levels, it has recently been demonstrated that these adducts can be formed independently of lipid peroxidation through direct DNA oxidation [62]. Low correlations between dG-MDA levels and lipid peroxidation rates may also arise if MDA adduct levels are low enough to prevent complete repair [63], and it has further been suggested that the relationship between circulating MDA concentrations and MDA adducts may be relatively weak because MDA is generated predominantly in cell membranes, some distance from cellular DNA [64].

Limitations of the present study include small sample size, which reduced our ability to detect interaction effects. Another limitation of the study was that catecholestrogen levels were not measured, therefore preventing examination of individual catecholestrogen levels along with levels of oxidative stress and breast density.

Conclusion

Results from the present study suggest that CYP1A2 activity is related to mammographic density levels and oxidative stress, but that the nature of the relationship depends on menopausal status. These findings provide further evidence that variation in the level of activity of enzymes involved in estrogen metabolism is related to breast density levels and potentially breast cancer risk [65].

Competing interests

None declared.

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