

Aromatase and breast cancer susceptibility

N M Probst-Hensch^{1,3}, S A Ingles¹, A T Diep¹, R W Haile¹,
F Z Stanczyk², L N Kolonel⁴ and B E Henderson¹

¹Department of Preventive Medicine, University of Southern California/Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033-0800, USA

²Women's and Children's Hospital, University of Southern California, Los Angeles, California 90033-0800, USA

³Institute of Social and Preventive Medicine of the University of Basel, CH-4051 Basel, Switzerland

⁴University of Hawaii at Manoa, Honolulu, Hawaii, USA

(Requests for offprints should be addressed to N M Probst-Hensch, Institute of Social and Preventive Medicine of the University of Basel, Steinengraben 49, CH-4051 Basel, Switzerland)

Abstract

Based on experimental and epidemiological evidence it is hypothesized that estrogen increases breast cancer risk by increasing mitotic activity in breast epithelial cells. Aromatase is crucial to the biosynthesis of estrogens and may therefore play a role in breast cancer development. Supporting data for an etiological role of aromatase in breast tumor biology are several-fold. First, the association between weight and postmenopausal breast cancer risk may be mediated by aromatase. Secondly, a pilot study found a higher aromatase expression in normal breast adipose tissue from breast cancer cases as opposed to healthy women. Thirdly, experimental data in animals suggest that aromatase activity predisposes mammary tissue to preneoplastic and neoplastic changes. In a multiethnic cohort study conducted in Los Angeles and on Hawaii we investigated (i) whether the plasma estrone to androstenedione (E1/A) ratio in different ethnic groups was associated with ethnic differences in breast cancer incidence, and (ii) whether genetic variation in the *CYP19* gene encoding the P450 aromatase protein was associated with breast cancer risk. The age- and weight-adjusted ethnic specific E1/A ratios $\times 100$ among women without oophorectomy were 7.92 in African-Americans, 8.22 in Japanese, 10.73 in Latinas and 9.29 in non-Latina Whites ($P=0.09$). The high E1/A ratio in Latina women was not associated with a high breast cancer incidence; in fact Latina women had the lowest breast cancer incidence in the cohort observed so far. We found no consistent association of an intronic (TTTA)_n repeat polymorphism with breast cancer risk in different ethnic groups. This polymorphism was not associated with differences in the plasma E1/A ratio in a way that would predict its functional relevance. We describe a newly identified TTC deletion in intron 5 of the *CYP19* gene that is associated with the (TTTA)_n repeat polymorphism. Neither this polymorphism, nor a polymorphism at codon 264 in exon VII of the *CYP19* gene, was associated with breast cancer. We did not identify any genetic variation in exon VIII in 54 African-American subjects. We identified rare genetic variants of unknown functional relevance in the promoter 1.4 of the *CYP19* gene in 3 out of 24 Latina women. Further investigation into the role of aromatase in breast cancer etiology is important, given that the potential use of aromatase inhibitors as breast cancer chemopreventives depends on these results.

Endocrine-Related Cancer (1999) 6 165-173

Introduction

Data from several research areas point to the involvement of sex hormones in the etiology of breast cancer. Laboratory studies have shown that estrogens control the growth of breast epithelial cells. Reducing estrogen

exposure affects the course of established breast cancer. Reproductive events and obesity have a substantial impact on a woman's lifetime risk of breast cancer, probably in part through their impact on the estrogen exposure of breast epithelial cells. Based on this evidence the

hypothesis was developed that high serum concentrations of endogenous estrogen, and specifically estradiol (E2), increase breast cancer risk (Henderson *et al.* 1982). The data from prospective studies assessing hormone levels and breast cancer risk in postmenopausal women have recently been combined in a systematic review and quantitative analysis by Thomas *et al.* (1997b). The authors in fact observed a statistically significant higher risk of breast cancer among women with higher levels of serum E2.

After menopause, estrogen in the circulation is predominantly estrone (E1), derived from the peripheral aromatization of androstenedione (Grodin *et al.* 1973). Plasma estrogen production is directly correlated with body weight (MacDonald *et al.* 1978), indicating that most of the postmenopausal, extraglandular aromatization of plasma androstenedione takes place in adipose tissue. The aromatization of androgens to estrogens is catalyzed by the enzyme aromatase P450 (Simpson *et al.* 1994). Aromatase activity thus has the potential to play a role in the etiology of breast cancer. Several lines of evidence support this hypothesis.

First, evidence that postmenopausal obesity as well as weight gain over the adult years are positively associated with postmenopausal breast cancer risk has been substantiated, especially for women who never used hormone replacement therapy (Huang *et al.* 1997). A positive association between adiposity and plasma estrogen levels in postmenopausal women has been reported quite consistently (Cauley *et al.* 1989, Kaye *et al.* 1991, London *et al.* 1991, Hankinson *et al.* 1995, Potischman *et al.* 1996, Thomas *et al.* 1997a, Madigan *et al.* 1998). It has been hypothesized, therefore, that the impact of obesity on breast cancer risk is mediated at least in part through the increased aromatization of androstenedione to E1 in the adipose tissue of obese women.

Secondly, data by Agarwal *et al.* (1996) suggest that breast cancer patients may have an inherently higher aromatase expression in breast adipose tissue when compared with healthy women. They studied 9 women undergoing reduction mammoplasty and 18 breast cancer patients undergoing mastectomy. Non-tumor bearing adipose samples from mastectomies expressed significantly more aromatase than adipose tissue from reduction mammoplasty patients, a difference that is unlikely to be due to the tumor's influence on aromatase expression.

Thirdly, Bulun *et al.* (1996) showed that in the human breast the distribution pattern of aromatase P450 transcripts and adipose fibroblasts, the primary extraglandular site of aromatase P450 expression, correlates well with the most common and least common sites of carcinoma in the breast, the outer and inner regions respectively.

Fourthly, experimental data indicate an etiological role of aromatase activity in the development of breast tumors in animals. The integration site 5 of the mouse mammary tumor virus (MMTV) is within the aromatase gene. Transgenic mice that overexpress int-5/aromatase under the control of MMTV enhancer/promoter have mammary tissue predisposed to preneoplastic changes (Tekmal *et al.* 1996). Aromatase inhibitors are successful in preventing mammary tumors in experimental animals (Rao *et al.* 1985, Lubet *et al.* 1994, Moon *et al.* 1994, Gunson *et al.* 1995, Grubbs *et al.* 1996).

If the role of aromatase activity in breast cancer etiology was substantiated, aromatase inhibitors, currently used in the treatment of breast cancer, would deserve consideration as potential chemopreventives for breast cancer (Kelloff *et al.* 1998). To gain further insight into the role of aromatase activity in breast cancer development we investigated whether ethnic differences exist in plasma E1 to androstenedione (E1/A) ratios, a surrogate measure for aromatase activity that would correlate with ethnic differences in breast cancer incidence. In addition, pilot data on the association of breast cancer risk with genetic variation in the *CYP19* gene encoding the P450 aromatase are presented.

Materials and methods

Study population

In 1993, we initiated a cohort study of individuals aged 40-75 years (Kolonel *et al.* 1999). The cohort consists of 215 251 men and women living in the states of Hawaii and California (primarily Los Angeles county) and being mainly of African-American, Japanese, Latino and non-Latino White ancestry. The cohort was mainly accessed from the drivers' license files over the period from 1993 to 1996. Participants completed a 26-page, self-administered mail questionnaire that elicited information about diet, demographic variables, medical history, personal habits (e.g. smoking, drinking), physical activity, and, for women, reproductive history. Overall response rates (after three mailings) were 25.5% in African-American women, 51.3% in Japanese women, 21.3% in Latina women and 47.0% in non-Latina White women. Compared with the US Census for the entire population of the study areas, the cohort is somewhat more educated than the general population, but all levels of education are comparatively well represented. The correspondence in marital status between the cohort members and the US Census is high.

All respondents are being followed for incident cancers by passive linkage with Surveillance, Epidemiology and End Results registries and periodic matches of the cohort to the National Death Index, and the Voters' Registration and Death Certificate files in Hawaii and

California. In addition, active follow-up on cohort members through periodic newsletters and special mailings will be maintained.

In both locations, biological samples (blood and urine) are being collected from cases of incident cancer of selected sites, and from an approximately 1.5% random sample of cohort members to serve as controls. Participation rate for the sample collection component in all populations has exceeded 70% so far.

Eligible controls for the analysis of blood hormone levels were female cohort members who had reported no history of cancer. Women who had permanently stopped bleeding were considered postmenopausal if at least one of the following criteria applied: age ≥ 55 ; both ovaries removed; no hysterectomy; follicle-stimulating hormone ≥ 40 mIU/ml. Women who reported hormone intake within 2 weeks before blood being drawn were excluded. Women on whom no information about hormone intake at the time of blood being drawn was available were excluded if they reported hormone intake at the baseline or at the follow-up interview. Based on these exclusion criteria all subjects had E1 levels < 75 pg/ml and E2 levels < 32 pg/ml. For all subjects included in the study we had complete information on E1, E2, androstenedione, age and body mass index (BMI). Included in the study were 66 African-American, 30 Japanese, 58 Latina and 39 non-Latina White women.

A random sample of incident breast cancers and controls from postmenopausal African-American, Japanese, Latina and non-Latina White cohort members was selected for preliminary genotyping analysis for a tetranucleotide repeat polymorphism in intron 5 of the *CYP19* gene encoding the cytochrome P450 aromatase enzyme. A subsample of women was used to screen for polymorphisms in exon VIII and promoter/exon I.4 region of the *CYP19* gene.

Consent has been obtained from each subject after full explanation of the purpose and nature of all procedures used and analyses performed with their biological specimens. The investigation was approved by the local ethical committee at the University of Southern California.

Laboratory methods

As samples were collected, blood components were separated and stored in 0.5 ml aliquots at -80 °C. DNA was purified from buffy coats of peripheral blood samples for all cases and controls using a rapid DNA preparation method (Talmud *et al.* 1991).

Plasma levels of androstenedione, E1 and E2 lpg/ml were measured by sensitive and specific RIA methods which were previously validated in our laboratory (Goebelsmann *et al.* 1973, 1979, Stanczyk *et al.* 1988, Cassidenti *et al.* 1992). Prior to quantification, the hormones were first extracted with hexane:ethyl acetate

(3:2) and then separated from interfering metabolites by use of Celite column partition chromatography. The intraassay and interassay coefficients of variation ranged between 5 and 10% and 10 and 15% respectively for the three analytes.

An intronic tetranucleotide repeat at bp 682 in intron 5 of the *CYP19* gene was described by Polymeropoulos *et al.* (1991). For detection of the polymorphism, PCR reactions were performed with one radioactively labeled and one unlabeled primer and products were visualized on modified acrylamide gels. The following primers were used: forward 5'-GCAGGTACTTAGTTAGCTAC-3', reverse 5'-TTACAGTGAGCCAAGGTCGT-3'. The forward primer with final concentration of 1.5 pmol per reaction was labeled with [γ - 33 P]ATP (New England Nuclear, Boston, MA, USA) to a specific activity of 6000 Ci/mmol using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) according to a standard protocol (Sambrook *et al.* 1989). PCR reactions were performed in large scale using 96-well microtiter plates. Approximately 10 ng genomic DNA per subject in a reaction mixture were amplified on a MJ Research Inc., (Watertown, MA, USA) programmable thermal controller PTC-100. At least two control samples from each allele that was found by genotyping were directly sequenced using an Amersham Thermosequenase kit (Amersham, Arlington Heights, IL, USA) for confirmation of the number of TTTA repeats.

To screen for polymorphisms in exon VIII of the *CYP19* gene, a non-isotopic RNase Cleavage Assay of MisMatch Detect II Kit (Ambion Inc., Austin, TX, USA) was used. Primers used for a two-stage nested PCR were: outer-sense 5'-TTTCCCATCTTCCAATTTG-3'; outer-antisense 5'-AGAAGAAATTGGTTTTTAAAGATGT-3'; nested-T7 promoter inner-sense 5'-TAATACGACTCACT ATAGGGCCCATCTTCCAATTT-3'; nested-SP6 promoter inner-antisense 5'-ATTTAGGTGACACTATAG GAAGAAGAAATTGGTTTTTAAAGATGT-3'.

To screen for polymorphisms in the I.4 promoter region, a 1149 bp region (from -786 to $+363$, relative to the transcription start site) was amplified using primers 5'-GATCATGCTACAGTGCATGAA-3' and 5'-TTCAGC TCCAAAGATAAGTTCC-3'. From this PCR product, several smaller overlapping segments were amplified using nested primers. Nested PCR products were treated with shrimp alkaline phosphatase and exonuclease (Amersham) and directly sequenced using the Amersham Thermosequenase kit.

Statistical analysis

The distribution of the E1/A ratio was markedly skewed. Formal statistical testing was therefore performed on logarithmically transformed values, and geometric mean values are presented. The ANOVA method was used to

compare the E1/A ratio between ethnic groups and between weight categories within ethnic groups. Weight tertiles were formed based on the weight distribution in the total study population. An ANCOVA method was used to compare the E1/A ratio between ethnic groups while adjusting for the potential effect of weight and age. Linear regression analysis was used to assess the linear association between the E1/A ratio and age or weight. All *P* values presented are two-sided (Ott 1988).

Results

Selected characteristics of the study population are shown in Table 1. African-American women were most likely to have their first child under the age of 21 (51%), whereas Japanese women were least likely to have their first child under the age of 21 (11%). Weight, height and BMI were highest in African-American women (79 kg, 164 cm, 29.4 kg/m²), and lowest in Japanese women (55 kg, 153 cm, 23 kg/m²). African-American women were most likely to have had at least one ovary removed (14.5% one ovary, 16.1% two ovaries, 3.2% unknown how many), and

Japanese women were least likely to have had at least one ovary removed (3.6% one ovary, 3.6% two ovaries).

In Table 2 we examined the ethnic distribution of the E1/A ratio×100. We observed no statistically significant differences in androstenedione levels among ethnicities (*P*=0.65 after adjustment for weight and age). The highest E1/A ratio×100 was observed among Latina women (9.68) as compared with 8.25 in African-American, 9.31 in Latina and 8.57 in non-Latina White women. Restriction of the analysis to subjects without oophorectomy revealed age- and weight-adjusted E1/A ratios×100 of 7.92 for African-American, 8.22 for Japanese, 10.73 for Latina and 9.29 for non-Latina White women (*P*=0.09). Ethnic-specific, age-adjusted breast cancer incidence rates (per 100000) observed in the cohort so far are 137.1 (African-Americans), 139.0 (Japanese) 84.3 (Latina) and 131.7 (non-Latina White).

Adjustment in Table 2 for BMI (kg/m²) or adiposity (kg/m^{1.5}) instead of weight did not substantially alter any of the findings presented. We also investigated the effect of reproductive variables on the E1/A ratio. Adjustment for whether or not a woman had ever been pregnant, the

Table 1 Characteristics of the study population by ethnicity

	African-American	Japanese	Latina	Non-Latina White
No. subjects	66	30	58	39
Mean age (years)	64	67	65	68
Mean height (cm)	164	153	159	159
Mean weight (kg)	79	55	71	67
Mean BMI (kg/m ²)	29.4	23.3	28.2	26.7
Parity (% nulliparous)	4.5	6.9	7.0	5.1
Age (years) at first birth				
≤ 20 (%)	50.8	11.1	31.4	16.7
20–30 (%)	41.0	77.8	64.7	66.6
>30 (%)	8.2	11.1	3.9	16.7
Age (years) at first period				
≤12 (%)	48.5	48.3	42.9	53.8
13–14 (%)	36.4	37.9	46.4	35.9
>14 (%)	15.2	13.8	10.7	10.3
Ovaries removed				
None (%)	66.1	92.8	83.3	88.8
One (%)	14.5	3.6	3.7	5.6
Two (%)	16.1	3.6	11.1	5.6
At least one, but not known how many (%)	3.2	0.0	1.9	0.0

Table 2 Geometric mean plasma E1/A ratio $\times 100$ in postmenopausal women: stratified by ethnicity

	African-American	Japanese	Latina	Non-Latina White	P-value ethnicity
E1/A $\times 100$					
Age and weight adjusted	8.25 (n=66)	9.31 (n=30)	9.68 (n=58)	8.57 (n=39)	0.65 ^a
Age and weight adjusted, no oophorectomy	7.92 (n=41)	8.22 (n=26)	10.73 (n=45)	9.29 (n=32)	0.09 ^b

^a P value for main effects of weight and age are 0.002 and 0.07 respectively.

^b P value for main effects of weight and age are 0.003 and 0.01 respectively.

age at menarche, the age at menopause, and the age at first birth in the ANCOVA did not substantially alter any of the results presented in Table 2.

The weight effect on hormone levels in different ethnic groups is presented in Table 3. Restriction of the analysis to women with intact ovaries did not alter the results. In African-American, Latina and White women the highest E1/A ratio was observed in the highest weight tertile. None of the Japanese women weighed 75 kg or more. Neither linear regression analysis nor ANOVA indicated a consistently statistically significant association between weight and the E1/A ratio in all ethnic groups.

Data on allele distribution for the intronic tetranucleotide repeat polymorphism among postmenopausal Japanese, African-American, Latina and White breast cancer cases and controls are presented in Table 4. We identified six alleles in our multiethnic study population with the numbers of TTTA repeats being 7, 8, 10, 11, 12 and 13. In African-Americans only, we observed three additional alleles that had a very low

frequency of less than 1% that were not further sequenced for determination of the repeat number. No single TTTA repeat allele was consistently more prevalent in breast cancer cases in all ethnic groups. Analysis of the data by genotype instead of alleles led to the same conclusion: no single genotype was consistently more prevalent among breast cancer cases in all ethnic groups, but the data became sparse. In African-American women, the largest ethnic group in our study, we also investigated the association of different genotypes with the plasma E1/A ratio, as a surrogate measure for phenotype in a subsample of control subjects (Table 5). Those genotypes with a higher prevalence in breast cancer cases as opposed to controls were not consistently associated with a high E1/A ratio.

In intron 5 we identified additional genetic variation in the vicinity of the (TTTA)_n repeat polymorphism. At 5' of the (TTTA)₇ repeat polymorphism we observed a TTC deletion. The deletion was present in 36% of African-American, 22% of Japanese, 34% of Latina and 35% of

Table 3 Geometric mean E1/A ratio $\times 100$ by weight tertile^a: stratified by ethnicity

	African American		Japanese		Latina		Non-Latina White	
	n	x _g	n	x _g	n	x _g	n	x _g
E1/A $\times 100$								
Weight tertile 1 (<62 kg)	8	6.41	25	8.40	16	10.31	15	7.94
Weight tertile 2 (<75 kg)	21	7.81	5	7.25	22	7.90	12	6.50
Weight tertile 3 (≥ 75 kg)	37	10.56	0	—	20	11.46	12	12.44
P value (ANOVA) ^b	—	0.06	—	0.50	—	0.17	—	0.03
P value (regression) ^c	—	0.01	—	0.70	—	0.38	—	0.17

^a Tertile formation based on weight distribution in the total study population.

^b P value based on one-way ANOVA.

^c P value based on linear regression of log(E1/A) on weight.

Table 4 Allele distribution of the (TTTA)_n repeat polymorphism in intron 5 of the CYP19 gene in postmenopausal breast cancer cases and controls: stratified by ethnicity

Allele (number of TTTA repeats)	African-American				Japanese				Latina				Non-Latina White			
	Case		Control		Case		Control		Case		Control		Case		Control	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
13	0	0	1	1	0	0	1	1	0	0	0	0	1	1	0	0
12	2	1	10	5	0	0	2	2	2	2	1	1	1	1	3	2
11	25	18	21	10	3	15	34	42	33	27	58	29	34	35	42	32
10	0	0	2	1	0	0	2	2	1	1	3	2	2	2	4	3
8	7	5	16	7	0	0	0	0	4	3	6	3	11	11	17	13
7	108	76	157	76	17	85	43	53	82	67	130	65	49	50	66	50
Total	142		207		20		82		122		198		98		132	

White control subjects, so that the intronic sequence around the (TTTA)_n repeat polymorphism read 5'...AATC*TTTTTTGTCTATGAATGTGC-CTTTTTT GAAATCATATTTTTTAAAATAT[TTTA]₇TTGAG....3' where * indicates the location of the TTC deletion. This variation was not observed in subjects with a number of TTTA repeats different from seven, other than in three African-American subjects with eight TTTA repeats and the TTC deletion. We did not find this variation to be associated consistently with breast cancer risk in all ethnic groups.

Screening for polymorphisms in exon VIII did not reveal any genetic variation among African-American women we studied (27 control and 25 breast cancer cases).

Screening for polymorphisms in the promoter/exon I.4 among 16 control and 8 breast cancer subjects of Latina origin revealed a few rare variants. One woman was a T/C heterozygote at position -771 (numbering from the start site of transcription) and an A/T heterozygote at position -757. One woman was an A/G heterozygote at position -616. A third woman was a heterozygote at positions

-236 (A/G) and -336 (A/G). These three Latina women exhibiting genetic variation were breast cancer cases.

Discussion

The E1/A ratio was measured to reflect aromatase activity. This ratio is expected to reflect the peripheral aromatization of androstenedione, since the clearance rates of both E1 and androstenedione are similar (Judd *et al.* 1980). We did not observe any statistically significant differences in the E1/A ratio between ethnic groups. We observed the highest E1/A ratio in Latina women, a difference that became almost statistically significant among women without oophorectomy. This finding did not correlate with ethnic differences in breast cancer incidence. In fact the age-adjusted breast cancer incidence rate in the multiethnic cohort study was lowest for Latina (84 per 100 000), followed by 137 per 100 000 for African-American, 138 per 100 000 for non-Latina White and 139 per 100 000 for Japanese women. This observation raises the following questions. First, does the

Table 5 (TTTA)_n repeat polymorphism genotype in postmenopausal African-American women: association with geometric mean E1/A×100

Genotype (number of TTTA repeats)	Genotype prevalence in breast cancer cases: controls (%)	n _{phenotyped controls}	Geometric mean plasma
			E1/A×100
7/7	64:57	36	7.79
7/8	4:12	6	10.42
7/10	0:2	2	11.32
7/11	20:16	12	11.36
7/12	1:7	6	11.92
8/11	6:4	1	3.03

E1/A ratio measured in the plasma of postmenopausal women properly reflect aromatase activity overall or in the relevant target tissue, and at the relevant time window? Second, is the impact of aromatase activity alone on breast cancer risk too subtle to explain ethnic differences in breast cancer incidence, given the multifactorial origin of breast cancer? Third, does aromatase activity not matter with regard to breast cancer etiology? It is conceivable that the interconnection between obesity, plasma estrogen levels, and breast cancer risk are merely associated with the interconversion of androstenedione to E1, but not mediated through aromatase activity.

In agreement with previous studies we found an indication of a positive association between weight and the E1/A ratio in ethnic groups other than Japanese. We did not observe a weight effect on the E1/A ratio in Japanese women. It is conceivable that Japanese women in our study lacked degrees of obesity that substantially influence the interconversion of androstenedione to E1. Our results as well as results presented by Madigan *et al.* (1998) indicate a non-linear weight effect on estrogen levels, with a substantial effect mainly observable at weights 80 kg and higher.

The role of aromatase in the association between weight and postmenopausal breast cancer risk must be further investigated. Exactly how obesity influences the aromatization rate is also unclear. Cleland *et al.* (1985) did not observe any correlation between aromatase activity of freshly prepared adipose tissue stromal cell suspensions and body weight of young women. Since obesity increases postmenopausal breast cancer risk, and if this effect is mediated by aromatase activity, Cleland's data potentially indicate that obesity reflects an increase in the amount of adipose tissue, and thus aromatase activity in the body overall, rather than reflecting an impact on local aromatase activity in breast adipose tissue.

Unlike previous studies (Hemsell *et al.* 1974, Cleland *et al.* 1985, Bulun & Simpson 1994) we did not consistently observe an increase in the E1/A ratio with age in all ethnic groups (data not presented). Conceivably, this is due to the fact that our study population was postmenopausal. There is graphical indication in the data from previous studies that the effect on aromatase activity and expression attributed to age may in fact be a menopause effect (Hemsell *et al.* 1974, Cleland *et al.* 1985, Bulun & Simpson 1994). Although Cleland *et al.* (1985) found that aromatase activity in adipose stromal cells from five women under the age of 45 who had undergone a previous surgically induced menopause, and who were not being treated with estrogens, were not significantly different from an age-matched group of premenopausal women, the issue of the menopause impact on aromatase activity deserves further investigation given the small sample size of this study.

We believe our plasma hormone measurements accurately reflect hormone concentrations in blood. Single hormone measurements in postmenopausal women have been shown to be reproducible over a 2-3 year period. They should suffice to rank subjects with regard to long-term hormone concentrations (Micheli *et al.* 1991, Hankinson *et al.* 1995). Unlike previously reported data (Thomas *et al.* 1997b), we did not find any relationship between duration of blood sample storage and hormone concentrations. Although blood was not consistently sampled at the exact same time of the day, and adrenal androstenedione excretion is subject to a circadian rhythm with peak excretion during the very early morning hours, we do not expect this problem to influence our finding of racial differences in androstenedione and E1/A ratio. Madigan *et al.* (1998) found no significant differences in serum androstenedione levels depending on sample collection time.

Assessment of genetic variation in the *CYP19* gene is another way to investigate the role of aromatase activity in breast cancer etiology. In principle, genotyping may offer the advantage of potentially reflecting a susceptibility history independent of short-term fluctuations in aromatase activity.

In agreement with previous data (Watanabe *et al.* 1997), we did not find an association between a C→T point mutation in exon VII, codon 264 and breast cancer risk (data not presented).

We investigated the role of an intronic tetranucleotide repeat polymorphism in the *CYP19* gene. In a group of non-Latina White women Kristensen *et al.* (1998) found five different alleles among 366 breast cancer cases and 252 controls. It is theoretically conceivable that the (TTTA)_n repeat affects transcriptional activation of *CYP19* (Kristensen *et al.* 1998). However several observations in our study are counter to this hypothesis. First, the allele with 12 repeats was statistically significantly more frequent in their Caucasian population, with a prevalence of 4% in breast cancer cases and 2% in controls. But in our White subjects this allele was more frequent in controls (3%) as opposed to cases (1%). Secondly, none of the six alleles observed in our study population was consistently more prevalent in cases in all ethnic groups. Thirdly, in an attempt to associate genotype with phenotype (E1/A ratio) we did not observe those genotypes with a higher prevalence in breast cancer cases to be associated with a high E1/A ratio. The role of the (TTTA)_n repeat polymorphism as well as the newly identified TTC deletion in a subset of subjects with the seven-repeat allele deserves further investigation before an association with breast cancer can be assigned to it.

Because with regard to breast cancer susceptibility we are interested in subtle rather than drastic effects of genetic variation on aromatase activity, and in order to detect

functionally more relevant variants of the *CYP19* gene, we shifted our screening focus to the promoter region of the aromatase gene. To look for functionally more relevant variants of the *CYP19* gene we started screening for further polymorphisms. We screened the region of the promoter I.4 and unspliced exon I.4, given the relevance of promoter I.4 in driving aromatase expression in normal adipose tissue, including breast adipose tissue (Zhao *et al.* 1995b, Agarwal *et al.* 1996). In our sample of Latina women, we only identified rare variants of questionable relevance to breast cancer risk, given their low prevalence, and the fact that they are not located in potentially important regulatory regions identified thus far in this promoter (Zhao *et al.* 1995a).

Given the evidence suggesting the role of aromatase activity in the etiology of breast cancer, research into this issue must continue. We plan to continue systematic screening for polymorphisms and will next focus on the promoter I.3 and II regions, which were found to be relevant to aromatase expression in the adipose tissue in breast cancer patients (Agarwal *et al.* 1996, Zhao *et al.* 1997, Zhou *et al.* 1997). The relevance of the genetic control of steroid biosynthesis to serum hormone levels and thus breast cancer risk has recently been evidenced for a cytochrome P450c17 α gene polymorphism (Spencer Feigelson *et al.* 1998). It is likely that this polymorphism acts in concert with genetic variation in the activity of other enzymes in the steroid biosynthesis pathway, for example aromatase.

Acknowledgements

This work was supported in part by the University of California Breast Cancer Research Program Grant No. 21B-0019 and by funds from the Public Health Service (National Cancer Institute). Grants RO1 CA 54281 and RA 63464.

References

Agarwal VR, Bulun SE, Leitch M, Rohrich R & Simpson ER 1996 Use of alternative promoters to express the aromatase cytochrome P450 (*CYP19*) gene in breast adipose tissue of cancer-free and breast cancer patients. *Journal of Clinical Endocrinology and Metabolism* **81** 3843-3849.

Bulun SE & Simpson ER 1994 Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. *Journal of Clinical Endocrinology and Metabolism* **78** 428-432.

Bulun SE, Sharda G, Rink J, Sharma S & Simpson ER 1996 Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. *Journal of Clinical Endocrinology and Metabolism* **81** 1273-1277.

Cassidenti DL, Pike MC, Vijod AG, Stanczyk FZ & Lobo RA 1992 A reevaluation of estrogen status in postmenopausal women who smoke. *American Journal of Obstetrics and Gynecology* **166** 1444-1448.

Cauley JA, Gutai JP, Kuller LH, LeDonne D & Powell JG 1989 The epidemiology of serum sex hormones in postmenopausal women. *American Journal of Epidemiology* **129** 1120-1131.

Cleland WH, Mendelson CR & Simpson ER 1985 Effects of aging and obesity on aromatase activity of human adipose cells. *Journal of Clinical Endocrinology and Metabolism* **60** 174-177.

Goebelsmann U, Horton R, Mestman JH, Arce JJ, Nagata Y & Nakamura RM 1973 Male pseudohermaphroditism due to testicular 17 β -hydroxysteroid dehydrogenase deficiency. *Journal of Clinical Endocrinology and Metabolism* **36** 867-879.

Goebelsmann U, Bernstein GS, Gale JA, Kletzky OA, Nakamura RM & Coulson AH 1979 Serum gonadotropin, testosterone, estradiol and estrone levels prior to and following bilateral vasectomy. In *Vasectomy: Immunologic and Pathophysiologic Effects in Animals and Men*, pp 165-175. Eds JH Lepow & R Crozier. New York: Academic Press.

Grodin JM, Siiteri PK & MacDonald PC 1973 Source of estrogen production in postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **36** 207-214.

Grubbs CJ, DeCoster R, Browden CR, Steele VE, Whitaker LM, Swanson SM, Kelloff GJ & Lubet R 1996 Vorozole, an aromatase inhibitor, as a chemopreventive agent in methylnitrosourea (MNU)-induced mammary cancer model (Abstract 1867). *Proceedings of the American Association for Cancer Research* **37**.

Gunson DE, Steele VE & Chau RY 1995 Prevention of spontaneous tumours in female rats by fadrozole hydrochloride, an aromatase inhibitor. *British Journal of Cancer* **72** 72-75.

Hankinson SE, Willett WC, Manson JAE, Hunter DJ, Colditz GA, Stampfer MJ, Longcope C & Speizer FE 1995 Alcohol, height, and adiposity in relation to estrogen and prolactin levels in postmenopausal women. *Journal of the National Cancer Institute* **87** 1297-1302.

Hemsell DL, Grodin JM, Brenner BF, Siiteri PK & MacDonald PC 1974 Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. *Journal of Clinical Endocrinology and Metabolism* **38** 476-479.

Henderson BE, Ross RK, Pike MC & Casagrande JT 1982 Endogenous hormones as a major factor in human cancer. *Cancer Research* **42** 3232-3239.

Huang Z, Hankinson SE, Colditz GA, Stampfer MJ, Hunter DJ, Manson JAE, Hennekens CH, Rosner B, Speizer FE & Willett WC 1997 Dual effects of weight and weight gain on breast cancer risk. *Journal of the American Medical Association* **278** 1407-1411.

Judd HL, Davidson BJ, Frumar AM, Shamonki IM, Lagasse LD & Ballon SC 1980 Serum androgens and estrogens in postmenopausal women with and without endometrial cancer. *American Journal of Obstetrics and Gynecology* **136** 859-871.

- Kaye SA, Folsom AR, Soler JT, Prineas RJ & Potter JD 1991 Associations of body mass index and fat distribution with sex hormone concentrations in postmenopausal women. *International Journal of Epidemiology* **20** 151-156.
- Kelloff GJ, Lubet RA, Lieberman R, Eisenhauer K, Steele VE, Crowell JA, Hawk ET, Boone CW & Sigman CC 1998 Aromatase inhibitors as potential cancer chemopreventives. *Cancer Epidemiology, Biomarker and Prevention* **7** 65-78.
- Kolonel LN, Henderson BE, Hankin JH, Nomura AMY, Wilkens LR, Pike MC, Stram DO, Monroe KR, Earle ME & Nagamine FS 1999 A multiethnic cohort in Hawaii and Los Angeles: baseline characteristics. *American Journal of Epidemiology* (In Press).
- Kristensen VN, Andersen TI, Lindblom A, Erikstein B, Magnus B & Borresen-Dale AL 1998 A rare *CYP19* (aromatase) variant may increase the risk of breast cancer. *Pharmacogenetics* **8** 43-48.
- London S, Willett W, Longcope C & McKinlay S 1991. Alcohol and other dietary factors in relation to serum hormone concentrations in women at climacteric. *American Journal of Clinical Nutrition* **53** 166-171.
- Lubet RA, Steele VE, Casebolt TL, Eto I, Kelloff GJ & Grubbs CJ 1994 Chemopreventive effects of the aromatase inhibitors vorozole (R-83842) and 4-hydroxyandrostenedione in the methylnitrosourea (MNU)-induced mammary tumor model in Sprague-Dawley rats. *Carcinogenesis* **15** 2775-2780.
- MacDonald PC, Edman CD, Hemsell DL, Porter JC & Siiteri PK 1978. Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer. *American Journal of Obstetrics and Gynecology* **130** 448-454.
- Madigan MP, Troisi R, Potischman N, Dorgan JF, Brinton LA & Hoover RN 1998 Serum hormone levels in relation to reproductive and lifestyle factors in postmenopausal women (United States). *Cancer Causes and Control* **9** 199-207.
- Micheli A, Muti P, Pisani P, Secreto G, Rechione C & Totis A 1991 Repeated serum and urinary androgen measurements in premenopausal and postmenopausal women. *Journal of Clinical Epidemiology* **44** 1055-1061.
- Moon RC, Steele VE, Kelloff GJ, Thomas CF, Detrisac CJ, Mehta RG & Lubet RA 1994 Chemoprevention of MNU-induced mammary tumorigenesis by hormone response modifiers: toremifene, RU 16117, tamoxifen, aminoglutethimide and progesterone. *Anticancer Research* **14** 889-893.
- Ott L (Ed.) 1988 *An Introduction to Statistical Methods and Data Analysis*, edn 3. Boston: PWS-Kent Publishing Company.
- Polymeropoulos MH, Xiao H, Rath DS & Merrill CR 1991 Tetranucleotide repeat polymorphism at the human aromatase cytochrome P-450 gene (*CYP19*). *Nucleic Acids Research* **19** 195.
- Potischman N, Swanson CA, Siiteri P & Hoover RN 1996 Reversal of relation between body mass and endogenous estrogen concentrations with menopausal status. *Journal of the National Cancer Institute* **88** 756-758.
- Rao AR, Das MG & Das P 1985 Inhibitory action of aminoglutethimide on DMBA-induced mammary carcinogenesis. *Oncology* **42** 119-121.
- Sambrook J, Fritsch EF & Maniatis T 1989 Labeling the 5' terminus of DNA with bacteriophage T4 polynucleotide kinase. In *Molecular Cloning: A Laboratory Manual*, edn 2. Eds J Sambrook, EF Fritsch & T Maniatis. Plainview, New York: Cold Spring Harbor Laboratory Press.
- Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR & Bulun SE 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342-355.
- Spencer Feigelson H, Shames LS, Pike MC, Coetzee GA, Stanczyk FZ & Henderson BE 1998 Cytochrome P450c17 α gene (*CYP17*) polymorphism is associated with serum estrogen and progesterone concentrations. *Cancer Research* **58** 585-587.
- Stanczyk FZ, Shoupe D, Nunez V, Macias-Gonzales P, Vijod MA & Lobo RA 1988 A randomized comparison of nonoral estradiol delivery in postmenopausal women. *American Journal of Obstetrics and Gynecology* **159** 1540-1546.
- Talmud P, Tybjaerg-Hansen A, Bhatnagar D, Mbewa A, Miller JP & Durrington P 1991 Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolemia. *Atherosclerosis* **89** 137-141.
- Tekmal RR, Ramachandra N, Gubba S, Durgam VR, Mantione J, Toda K, Shizuta Y & Dillehay DL 1996 Overexpression of *int-5/aromatase* in mammary glands of transgenic mice result in the induction of hyperplasia and nuclear abnormalities. *Cancer Research* **56** 3180-3185.
- Thomas HV, Key TJ, Allen DS, Moore JW, Dowsett M, Fentiman I & Wang DY 1997a Re: reversal of relation between body mass and endogenous estrogen concentrations with menopausal status. *Journal of the National Cancer Institute* **89** 396-397.
- Thomas HV, Reeves GK & Key TJ 1997b Endogenous estrogen and postmenopausal breast cancer: a quantitative review. *Cancer Causes and Control* **8** 922-928.
- Watanabe J, Harada N, Suemasu K, Higashi Y, Gotoh O & Kawajiri K 1997 Arginine-cysteine polymorphism at codon 264 of the human *CYP19* gene does not affect aromatase activity. *Pharmacogenetics* **7** 419-424.
- Zhao Y, Mendelson CR & Simpson ER 1995a Characterization of the sequences of the human *CYP19* (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes. *Molecular Endocrinology* **9** 340-349.
- Zhao Y, Nichols JE, Bulun SE, Mendelson CR & Simpson ER 1995b Aromatase P450 gene expression in human adipose tissue. *Journal of Biological Chemistry* **270** 16449-16457.
- Zhao Y, Agarwal VR, Mendelson CR & Simpson ER 1997 Transcriptional regulation of *CYP19* gene (aromatase) expression in adipose stromal cells in primary culture. *Journal of Steroid Biochemistry and Molecular Biology* **61** 203-210.
- Zhou D, Zhou C & Chen S 1997 Gene regulation studies of aromatase expression in breast cancer and adipose stromal cells. *Journal of Steroid Biochemistry and Molecular Biology* **61** 273-280.