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PRINCIPAL INVESTIGATOR: Paola C. Muti, M.D.

CONTRACTING ORGANIZATION: New York State University at Amherst
Amherst, NY 14228-2567

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14. **ABSTRACT**
The prevalence of breast cancer is greatest in industrialized regions and exposure to light at night has been proposed as a potential risk factor. Modulation of melatonin secretion by light has been implicated in the caupathway linking exposure to light and breast cancer risk. Recent evidence indicates that melatonin is a naturaoncostatic agent capable of functioning through a variety of anti-proliferative, anti-oxidative, and immunostimulatory mechanisms. We conduct a study to investigate the association of prediagnostic melatonproduction and subsequent breast cancer risk in a prospective cohort study, the Italian ORDET study. Thus, prediagnostic melatonin production will be measured as urine levels of the 6-hydroxymelatonin sulphate (6-OHMS), its primary enzymatic metabolite, in 12-hour urine (overnight) collection. The study will be conducas a nested case-control study. We expect 533 breast cancer cases among cohort members during the 17 year follow-up period. Four controls will be matched to each case on age, menopausal status, recruitment center a time of recruitment for a total number of 2,132 control subjects. This study would be the first one analyzing potential effect of melatonin on breast cancer risk. It will provide important data on risk factors that are likely key to the development of this disease at great public health impact.

15. SUBJECT TERMS
Breast cancer, Melatonin, Epidemiological Study

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INTRODUCTION

Melatonin (N-acetyl-5methoxtryptamine) is synthesized and released by the pineal gland in response to darkness. Thus, melatonin displays a strong variation during the 24-hour period: its serum levels are low during the daylight hours and high at night. The health effects of chronic alteration of this circadian rhythm in humans have received relatively little attention. There is strong evidence indicating a role of melatonin as natural oncostatic substance (Blansk, 1993). Consistent experimental evidence, from both *in vitro* and *in vivo* studies, identified specific anti-carcinogenic functions of melatonin such as the anti-proliferation, anti-oxidation, and immunostimulation functions (Brzezinski, 1997; Panzer and Viljoen, 1997; Reiter et al, 1997).

Environmental factors that reduce nocturnal exposure to melatonin may increase breast cancer risk by increasing levels of estrogens, by increasing exposure to oxidative stress and by reducing immune function (Cohen et al, 1978; Stevens, 1987). Nighttime plasma melatonin is reported to be lower in women affected with estrogen-receptor-positive breast cancer in comparison with women affected by other pathologies (Tamarkin et al, 1989). Melatonin was also lower in breast cancer cases than in women with benign breast disease (Bartsh et al, 1989). We are conducting a study to evaluate the association of melatonin with breast cancer using data from a prospective cohort study in which several sources of possible biomarker variability have been controlled by study design. We measure pre-diagnostic urine levels of the main melatonin metabolite, **6-OHMS**, in urine stored at –80° C during the 17 year follow-up period. At its completion, the study will allow us to investigate the role of prediagnostic melatonin as a potential important factor underlying the association between environmental and life-style factors and breast cancer.

BODY OF REPORT

During the first budget year, study protocols were developed and discussed. Major issues were the test of the reliability of the **6-OHMS** determinations and the identification and selection of all breast cancer cases and the related controls, the selection of the samples within the biological specimen bank of the cohort study, and the preparation of the computer database.

Reliability of the 6-ohms determinations: Before beginning the dosage of Endogenous 6-Sulfatoxymelatonin (aMT6S) in the urine samples of ORDET study, we evaluated:

1. Reliability of determination
2. Coefficients of variation intra- and inter-assay
3. Comparison between different temperatures of storage

We used a competitive immunoassay (ELISA) (Bühlmann Laboratories AG, Switzerland).

1. Description of the Kit, reagents supplied, urine samples

EK-M6S (Bühlmann Laboratories AG, Switzerland) is competitive immunoassay designed to quantify aMT6s concentration in human urine.

The procedure consists in a competitive immunoassay using a capture antibody technique. In brief, urine samples are diluted 1:200 in the Kit's Incubation Buffer, then biotinylated aMT6s and anti-aMT6s antibody are added and left at 4°C for 3 hours. During incubation, aMT6s present in urine samples compete with biotinylated aMT6s for the binding sites of anti-aMT6s antibody, while the formed (bioinylated) aMT6S-antibodies complexes are captured by a second antibody coated on the wells. After washing, the Enzime Label (steptavidin conjugated to horseradish peroxidase) is added, which step to the aMT6s-biotin-antibody complexes captured on the coated wells. Unbound Enzyme Label is then removed by a second washing step and TMB substrate (tetramethylbenzidin) is added, and a colored product is formed in inverse proportion to the amount of aMT6s present in the samples. The color turns from blue to yellow after the addition of an acidic Stop Solution and can be measured at 450 nm, with background wavelength correction set at 600 nm, in a microtiter plate reader.

The kit is made up by:

1. Microtiter Plate
2. Wash Buffer Concentrate
3. Incubation Buffer
4. Concentrated aMT6S standard
5. Antiserum
6. Biotin Conjugate
7. Enzyme Label
8. TMB Substrate
9. Stop Solution
10. Quality controls at Low and High aMT6S concentration

To test the kit we used the following urine samples:

-28 samples of nocturnal urine of menopausal women stored both at -30° C and -80° C since 1990. These samples are analysed in two Kits of the same lot.

-3 nocturnal urine collected from three volunteers. Three volunteers gave one own urine sample, at arrived to the laboratory, urine samples were immediately filtered, divided in aliquots and frozen at – 30° C.

2. Reliability of determination

To assay reliability of determination we processed the quality controls supplied by the kit and we compared our results with the values declared by the enclosed data-sheet.
Data were reported in Table 1.

Table 1. Comparison between quality controls concentration obtained by the company and by our laboratory for 2 kits analysing.

Quality control	Company value (ng/ml)	Company CV%	Obtained value (ng/ml) Kit 1	Obtained value (ng/ml) Kit 2
QC Low	3,9	0,6	3,06	3,719
QC High	15,2	0,0	23,758	20,775

Conclusion

The company declared an "expected range" for quality controls of 1,2- 4,8 ng/ml for control Low and 11,5- 25,3 ng/ml for control High. Because our data are inside of these range, we supposed that these results reveal a good reliability of determination.

3 Coefficients of variation intra-assay

We analysed 3 nocturnal urine collected from three volunteers (named a, b, c) three times (named 1, 2, 3) in every kit. Results are reported in Table 2.

Table 2. Concentrations of aMT6s and relative coefficients of variation (CV%)

Sample	aMT6s (ng/ml) kit 1	aMT6s (ng/ml) kit 2	CV % Kit 1	CV % Kit 2
a1	32,003	27,548	3,7	1,8
a2	29,776	26,838		
a3	30,429	26,596		
b1	1,893	1,919	33,8	37,8
b2	1,192	1,43		
b3	1,019	0,858		
c1	12,577	8,705	14,5	9,8
c2	11,448	10,39		
c3	9,386	8,923		

Conclusion

Coefficients of variation intra-kit are similar to those declared by the company for the same concentrations of aMT6s.

4. Comparison between different temperatures of storage

We analysed 28 samples of nocturnal urine of menopausal women stored both at -30° C and -80° C since 1990. These samples are analysed in two Kits of the same lot. Samples of the same subject, stored at -30° C and -80° C, were measured in double wells in the same kit. In Table 3 are reported values of aMT6s.

Table 3. Values of aMT6s and relative urine volumes

Sample	aMT6s (ng/ml) -30°C	aMT6s (ng/ml) -80°C	Urine Volumes (ml)
1	12,23	30,46	470
2	14,885	16,998	910
3	12,894	20,424	450
4	5,243	9,806	590
5	6,515	7,001	1280
6	4,211	6,525	930
7	37,611	46,425	450
8	6,288	16,62	570
9	12,9	23,341	620
10	8,161	11,146	700
11	14,76	19,22	460
12	6,711	13,445	690
13	18,722	26,857	470
14	8,914	12,351	750
15	1,829	3,331	700
16	3,051	5,04	1050
17	10,964	15,172	640
18	9,569	11,85	590
19	11,07	10,308	1050
20	12,87	16,213	470

21	2,026	2,437	790
22	13,686	19,147	770
23	26,743	35,829	330
24	2,404	4,264	1030
25	7,564	9,784	350
26	6,59	14,96	680
27	9,915	16,535	280

We performed a Student *t* Test to compare mean values and we obtained a value of t=6,38496, that indicated they are statistically different (p<0,0001).

We also conducted a correlation analysis. The correlation coefficient (Pearson r) between storage at -30° C and -80°C was r= 0,929 (p<0,0001). The regression analysis equation was:

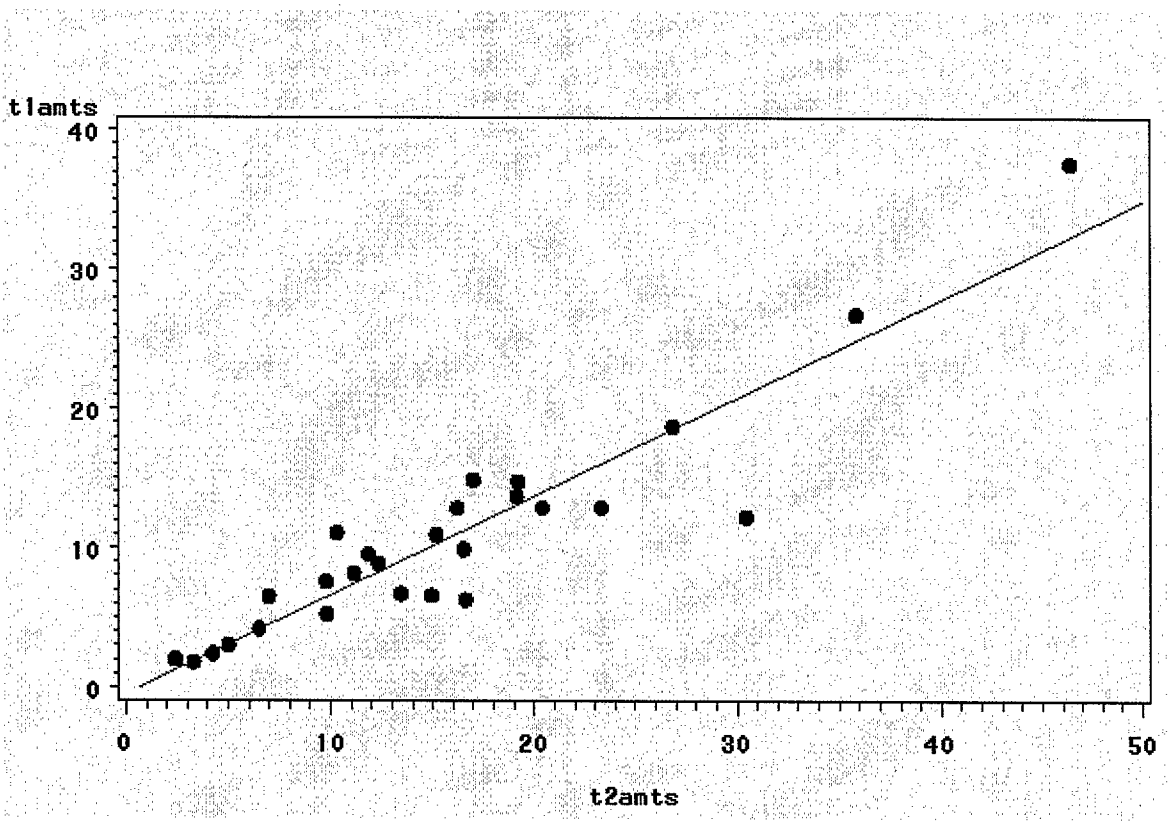
$$[aMt6s]_{-30^\circ C} = -0,4458 + 0,7059 * [aMt6s]_{-80^\circ C}.$$

The regression plot was as it follows:

Figure 1: Linear regression analysis

X-axis: aMT6S ng/ml -80°C

Y-axis: aMT6S ng/ml -30°C



Conclusion

Our data showed a constant difference between concentration of aMT6s in urine samples stored at -30° C and -80° C. Relationship between the values is linear and it is explained by the equation reported that outlined a concentration lower than 30% mean in the samples stored at -30°C. However, the values are well correlated with an r=0,929. This conclusion supports the hypothesis that the storage's temperature may affect degradation of aMT6s during storage's time.

The results of this study supported the decision to use the urine stored at -80° C as indicated in our protocol.

Retrieval of the samples and matching procedures: The retrieval of the samples from storage and their allocation to the appropriate batches and the related sample verification

was completed during this time period, so that each breast cancer case and the four related controls were handled in the same way and placed in the laboratory set. In each set included the breast cancer case and her four related controls. This set was placed in a laboratory box with nine rows and nine columns and each set was placed in the same row. The sample organization within the box allowed/allows the laboratory to conduct bioassays for cases and controls together in the same run being blind to the case-control status. During the same period, we developed detailed procedures for analytical determinations and quality control procedures and implemented at the Endocrinology Laboratory at the Italian NCI in Milan, responsible for the bioassays.

At the present time (May 24, 2000), out of the 2132 samples (533 samples from breast cancer cases, 1599 related control subjects), 320 have been already assayed. The analytical determinations for all the biomarkers will be completed in the next year.

Publications and Presentations

At this time, there are no results or publications coming directly from this grant because we have still to complete the study. However, Dr. Muti has published or has in press research on hormone related breast cancer.

In year 2004-2005, Dr. Muti has published other papers on hormones and breast cancer listed below:

1) Muti P. *The Role of Endogenous Hormones in the Etiology and Prevention of Breast Cancer: the Epidemiological Evidence.* Ann N Y Acad Sci. 2004; 1028:28-37

2) Rinaldi S, Toniolo P, **Muti P**, Lundin A, Zeleniuch-Jacquotte A, Akhmedkhanov A, Micheli A, Lenner P, Dossus L, Krogh V, Shore RL, Koenig KL, Riboli E, Stattin P, Berrino F, Hallmans G, Lukanova A, Kaaks R IGF and IGFBP3 and

breast cancer in young women: *A Pooled Reanalysis of three prospective studies European Journal of Cancer Prevention* (in press)

3) Carruba G, Cocciadiferro L, Bellavia V, Rizzo S, Tsatsanis C, Spandidos D, **Muti P**, Smith C, Mehta P, Castagnetta L. *Intercellular communication and human hepatocellular carcinoma* Ann N Y Acad Sci.1028:202-12

4) Bucca G , Carruba G , Saetta A , **Muti P**, Castagnetta L , Smith CP. *Gene expression profiling of human cancers* Ann N Y Acad Sci. 2004; 1028:28-37

5) Micheli A, **Muti P**, Secreto G, Krogh V, Meneghini E, Sieri S, Venturelli E, Pala V, Berrino F. *Endogenous sex hormones and subsequent breast cancer in premenopausal women.* Int J Cancer 2004: 112 (2):312-318

She also presents new study results from other conducted studies at the Annual Meeting of the American Association for Cancer Research (2005):

1) Fuhrmann B*, Barba M, Krogh V, Micheli A, Berrino F, Muti P. *Insulin resistance is associated with elevated circulating androgens in postmenopausal women: A potential pathway for breast cancer etiology* Annual Meeting American Association for Cancer Research, Anaheim, California, April 2005

2) Platek M, Freudenheim JL, Quick S, Nie J, Muti P, McCann S, Trevisan M,

Shields P, Edge **S** *Methylenetetrahydrofolate reductase (MTHFR) and risk of breast cancer: The Western New York Exposures and Breast Cancer Study (WEB Study)* Annual Meeting American Association for Cancer Research, Anaheim, California, April 2005

3) Barba M*, Mc Cann S, Stranges S, **Muti P**, Fuhrmann B, Trevisan M, Freudenheim J *Perinatal exposures and breast cancer risk: a case-control study* Annual Meeting American Association for Cancer Research, Anaheim, California, April 2005

Two of these studies have been submitted for publication. In addition, Dr. Muti has several other manuscripts submitted for publication on hormone and related factors and cancer.

In 2004, she published a paper on the relation between Growth Hormone and Prostate Cancer (Fuhrman B, Barba M, Schunemann HJ, Hurd T, Quattrin T, Cartagena R, Carruba G, **Muti P**. *Basal growth hormone concentrations in blood and the risk for prostate cancer: A case-control study.* Prostate. 2005- Jan 21) and a paper on alcohol consumption and risk of prostate cancer (Barba M, McCann SE, Schunemann HJ, Stranges S, Fuhrman B, De Placido S, Carruba G, Freudenheim JL, Trevisan M, Russell M, Nochajski T, **Muti P**. *Lifetime total and beverage specific - alcohol intake and prostate cancer risk: a case-control study* Nutr J. 2004; 3:23-29) . In both cases the first authors are young collaborators of Dr. Muti.

CONCLUSIONS

We have just begun the phase of hormone determinations for this grant; therefore, there are no conclusions to report at this time.

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APPENDIX

Appendix 1:

Platek M, Krogh V, Micheli A, Schünemann H, Sieri S, Meneghini E, Pala V, Browne R, Wilding G, Jo L. Freudenheim, Berrino F, Muti P. ***Serum fructosammine and subsequent risk of breast cancer: A Prospective Study.*** **Cancer Epidemiol Biomark Prev 2005 Jan;14(1):271-4**

Appendix 2:

Muti P. ***The Role of Endogenous Hormones in the Etiology and Prevention of Breast Cancer: the Epidemiological Evidence.*** **New York Academy of Science 1028:202-12, 2004**

Short Communication

Serum Fructosamine and Subsequent Breast Cancer Risk: A Nested Case-Control Study in the ORDET Prospective Cohort Study

Mary Platek,[1] Vittorio Krogh,[6] Andrea Micheli,[6] Richard Browne,[5] Elisabetta Meneghini,[6] Sabina Sieri,[6] Holger J. Schünemann,[2] Valeria Pala,[6] Maddalena Barba,[1] Gregory E. Wilding,[3] Franco Berrino,[6] and Paola Muti[4]

Departments of [1]Exercise and Nutrition Sciences, [2]Medicine, [3]Biostatistics, and [4]Social and Preventive Medicine, [5]Clinical Science Laboratory, University at Buffalo, State University of New York, Buffalo, New York; and [6]Epidemiology Unit, Instituto Nazionale Per lo Studio e la Cura dei Tumori, Via Venezian, Milan, Italy

Abstract

There is evidence that abnormal glucose metabolism may contribute to the risk of breast cancer. The measurement of markers of glucose metabolism could help to identify women at risk for breast cancer. Serum fructosamine is one such marker. In this study, we investigated whether prediagnostic serum fructosamine was associated with breast cancer. Between 1987 and 1992, 10,786 women ages 35 to 69 were recruited in Italy for a prospective study. Women with a history of cancer or on hormone therapy were excluded at baseline. Blood samples were collected after 12 hours fasting from all participants at recruitment. After 5.5 years of follow-up, 144 breast cancer cases were identified and four matched controls were selected from the cohort; serum fructosamine levels were measured in both groups at baseline. Adjusted odds ratios (OR) for the highest tertile of serum fructosamine compared to the lowest was 1.60 [95% confidence interval (CI), 0.95-2.73]. In premenopausal women, the OR was 1.58 (95% CI, 0.76-3.40) and in postmenopausal women, the OR was 1.60 (95% CI, 0.76-3.48). Serum fructosamine levels tended to be positively associated with breast cancer risk independent of menopausal status. (Cancer Epidemiol Biomarkers Prev 2005;14(1):271–4)

Introduction

There is increasing evidence that obesity and diabetes mellitus are associated with increased risk of breast carcinomas. Biological evidence provides support for a role of glucose and other factors related to glucose metabolism, such as insulin and C-peptide, in breast cancer development. It is known that glucose favors the selection of malignant cell clones and that the neoplastic cell extensively uses glucose for proliferation (1). Insulin has been shown to be a potent mitogenic agent (2). Insulin also induces a dose-dependent growth response in breast cancer cell lines and acts through the insulin receptor (3, 4). Furthermore, insulin may also play a role in tumor promotion by up-regulation of ovarian steroid secretion (5-7).

There is epidemiologic evidence supporting the relationship between abnormal glucose metabolism and breast cancer risk. There was an increase of breast cancer risk for women who had a diagnosis of diabetes mellitus at baseline in four prospective studies (8-11); however, a fifth did not corroborate the evidence. Furthermore, studies have shown a positive relationship between insulin and C-peptide levels and breast cancer incidence (12, 13). Additionally, variables related to insulin resistance, such as body mass index (BMI) and abdominal obesity have been related prospectively to breast cancer risk in postmenopausal women (14-16).

Given the evidence that supports a relationship between abnormal glucose metabolism and breast cancer risk, the measurement of markers of insulin resistance may help to identify women at high risk for breast cancer. Serum fructosamine, a product of protein glycation, may be suitable for the assessment of glucose metabolism in epidemiologic studies.

The spontaneous, nonenzymatic condensation of glucose and proteins initially produces an unstable ketoamine, which is generally referred to as fructosamine due to its structural similarities to fructose (17). Glycated albumin usually accounts for 80% of the glycated serum proteins (18, 19). Serum fructosamine is more strongly correlated with habitual intake of sugar ($r = 0.26$, $P = 0.05$) than glycated hemoglobin ($r = 0.001$, $P = 0.99$). Thus, fructosamine can also be considered an index of the chronic exposure to sugar intake (20).

The purpose of our study was to investigate the association between fructosamine and breast cancer cases in a prospective study. The primary hypothesis of the present study was that prediagnostic serum fructosamine, as a marker of glucose metabolism, was associated with subsequent breast cancer.

Materials and Methods

Between June 1987 and June 1992, 10,786 healthy women, ages 35 to 69 years, residents of Varese province in northern Italy, participated in a prospective study of hormones, diet and breast cancer risk: the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) study (20, 21). All members of the cohort were volunteers recruited from the general population. The total number of women recruited in the cohort represented ~7% of the general population of women in that age range in Varese province.

A major focus of the ORDET study was endogenous hormones and their relation to breast cancer risk. Therefore, several sources of hormone variability were controlled for by

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Requests for reprints: Paola Muti, Department of Social and Preventive Medicine, University at Buffalo, State University of New York, 270 Farber Hall, 3435 Main Street, 14214 Buffalo, NY. Phone: 716-829-2975; Fax: 716-829-2979. E-mail: muti@buffalo.edu

inclusion criteria and highly standardized conditions at blood drawing during the recruitment phase. Exclusion criteria included women with bilateral ovariectomy, those currently pregnant or breast-feeding, those on oral contraceptives, or hormone replacement therapy, or those affected by metabolic diseases and women with a previous history of cancer. At baseline, information on diet, reproductive history, family history of breast cancer, education, and occupational history were collected as well as anthropometric data.

After an average of 5.5 years of follow-up, the ORDET data were linked with the local Lombardy Cancer Registry (22, 23). These files were used to identify breast cancer cases. The regional municipal data of Varese residents was used to verify the vital status of the cohort members. Ten women were lost to follow-up, 37 women had been diagnosed with breast cancer before final enrollment in the cohort, and 4 were diagnosed with breast cancer *in situ*. Therefore, there were 10,735 women available for the study. Among these women, 89 died from causes other than breast cancer, and 144 were identified by the cancer registry as cases of invasive breast cancer (73 premenopausal and 71 postmenopausal at the time of recruitment). Postmenopausal status was defined as the absence of menstrual bleeding for at least 12 months before enrollment into the study.

Four control subjects were matched to each breast cancer case. Control subjects were randomly chosen from members of the cohort who did not develop breast cancer during the follow-up; matched to cases on age (±5 years), menopausal status, daylight saving period at recruitment, recruitment center, and date of recruitment (±89 days).

Blood samples were collected after at least 12 hours of fasting between 7:30 and 9:00 a.m. from all participants at recruitment. For premenopausal women, blood was collected in the luteal phase of the menstrual cycle, between the 20th and 24th day, where the first day of menses was counted as the first day of the ovarian cycle. All of the blood samples were processed and stored at −80°C until biochemical determinations were done.

Stored serum samples from breast cancer cases and related controls were handled identically and assayed together on the same day and in the same run. The laboratory personnel were blinded to case-control status. The control of analytic error was based on the inclusion of three standard samples. Serum glucose was determined on a Cobas Mira automated chemistry analyzer (Roche Diagnostic Systems, Indianapolis, IN). The intrabatch CV derived from the quality control serum included in the analytic runs was 2.5%. Fructosamine was determined using reagents, calibrators and controls from Sigma Diagnostics (St. Louis, MO) and application parameters for the Cobas Mira automated chemistry analyzer. The assay is a modification of the original method of Johnson and colleagues (24) where fructosamine (glycated serum protein) reduces nitroblue tetrazolium under alkaline conditions and forms a purple-colored formazan with an absorption maximum at 530 nm.

There were no stored serum specimens for 4 premenopausal breast cancer cases and 11 matched control subjects, and 7 postmenopausal cases and 18 controls. The final analysis included 69 premenopausal and 64 postmenopausal breast cancer cases and 265 premenopausal and 238 postmenopausal controls.

Statistical Analysis. Means and SD for serum glucose and fructosamine and for other risk factors were computed and compared for cases and control subjects with one-way ANOVA. In addition, we examined the difference between average fructosamine levels among smokers and nonsmokers. Pearson correlation coefficients were determined for the variables in the model and for serum glucose. The relationships between serum glucose and serum fructosamine were examined in premenopausal and postmenopausal controls using scatter plots. Serum fructosamine and BMI were collapsed into three and two categories, respectively, and these categories were used in the analyses. The cut-offs for serum fructosamine were based on the tertiles of the controls and the cut-off for BMI on the median of the controls to maintain the cut-off point used for BMI in the analysis of the original study. We estimated adjusted odds ratios and 95% confidence intervals (CI) for fructosamine using conditional logistic regression. We identified age, age at menarche, age at first birth, parity, BMI as potential covariates according to their potential biological relevance and logistic regression was used to control for these covariates. In the initial regression model, we examined all variables. We evaluated each covariate for confounding by removing each from the fully adjusted model. Age, age at menarche, age at first birth, parity, and BMI did not substantially modify the results. None of the potential covariates was a confounder of the association between breast cancer and fructosamine levels. Nevertheless, we included them in further analysis to provide fully adjusted estimates for comparison with those reported in the published literature, in particular, with the previous prospective cohort studies evaluating variables related to glucose metabolism (insulin and C-peptide) in relation to breast cancer risk. We also performed analyses after stratification by menopausal status.

Results

In Table 1, we report descriptive data on the study participants. Serum glucose levels were ~5% lower for premenopausal women than for postmenopausal women. Fasting glucose levels were significantly correlated with fructosamine levels among controls in the premenopausal women ($r = 0.129$, $P = 0.018$) and postmenopausal women ($r = 0.3019$, $P < 0.0001$). The correlation between fructosamine and glucose in postmenopausal women was stronger and this group of women on average had higher values for serum glucose as well. Figure 1 displays a scatter plot of glucose and fructosamine in controls.

Table 1. Baseline characteristics of breast cancer cases and controls by menopausal status

	Premenopausal women		Postmenopausal women	
	Cases (n = 69), mean (± SD)	Controls (n = 265), mean (± SD)	Cases (n = 64), mean (± SD)	Controls (n = 238), mean (± SD)
Age (y)	44.8 (5.0)	44.4 (4.8)	58.1 (5.5)	57.6 (5.3)
BMI (kg/m^2)	24.3 (3.9)	24.6 (4.6)	26.0 (4.0)	26.7 (4.2)
Age at menarche (y)	12.7 (1.5)	12.7 (1.5)	13.2 (1.6)	13.3 (1.6)
Age at first birth (y)	25.8 (4.5)	26.0 (4.4)	26.5 (5.1)	26.3 (4.6)
Number of children	1.8 (1.1)	1.9 (1.1)	1.8 (1.0)	2.1 (1.6)
Glucose (mg/dL)	81.9 (18.9)	78.6 (10.8)*	82.2 (23.2)	83.9 (34.0)
Fructosamine (mmol/L)	1.87 (0.13)	1.84 (0.13)	1.86 (0.13)	1.84 (0.13)
Smoking (yes/no)	25%/75%	42%/58%	31%/69%	21%/79%

*One-way ANOVA ($P < 0.05$): differences between cases and controls.

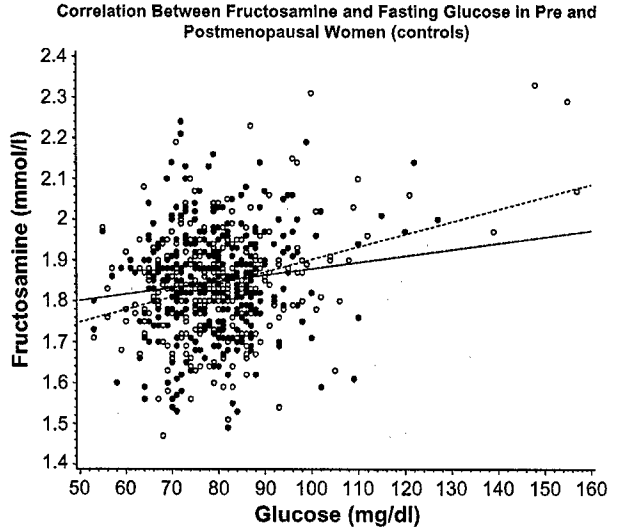

Figure 1. Correlation between fructosamine and fasting glucose in (● and —) premenopausal and (○ and - - -) postmenopausal women (controls).

In Table 2, the descriptive data for the categories of fructosamine levels are shown. Cases were more likely to have elevated fructosamine levels than control subjects prior to diagnosis (43% of premenopausal cases and 41% of postmenopausal cases were classified in the highest category).

Breast cancer risks in relation to categories of fructosamine are also shown in Table 2. Considering the group of women as a whole, women in the highest tertile of fructosamine had a nonsignificant 60% times higher risk of developing incident breast cancer (95% CI, 0.95-2.73). When we separated the sample in women who were in premenopausal and postmenopausal status at recruitment, we obtained similar risk estimates: 1.58 (95% CI, 0.76-3.40) and 1.60 (CI, 0.76-3.48), respectively. All the CIs included unity and there was no evidence of a linear dose-effect relation (P for trend = 0.32). Adjustment for glucose did not alter the odds ratio estimates.

Discussion

We observed a 60% risk increase for breast cancer in women with high levels of fructosamine. Although the results failed to reach conventional levels of statistical significance, our results are important because this is the first study investigating serum fructosamine in relation to breast cancer risk. Our results confirm previous work in which we observed an association of fasting glucose and breast cancer risk in premenopausal women and in heavier postmenopausal women. Although in the latter group of women, the relation was not statistically significant (25). Thus, there is accumulating evidence suggesting a relationship between impaired glucose metabolism and the outcome of breast cancer. Fructosamine is a biomarker of habitual sugar intake, a risk factor for the development of hyperinsulinemic insulin resistance and type 2 diabetes (26). The availability of such a marker for prospective studies would enhance epidemiologic research in this area.

The spontaneous, nonenzymatic condensation of glucose and proteins initially produces an unstable ketoamine, which is referred to as fructosamine (16). Fructosamine, then, is the generic name for plasma protein ketoamines. Available literature indicates that short- to intermediate-term glycemic control is best reflected by glycated albumin (27, 28). For individuals with hemoglobin variants, assaying fructosamine may be a superior method compared with measuring glycated hemoglobin (29-31).

Limitations of this investigation warrant consideration. First, although the results from this study suggest that fructosamine may be predictive of breast cancer, the confidence limits included unity. Second, our results are based on direct determination of fructosamine and many factors may have affected the study results. We were not able to make albumin determinations due to limitations in serum availability. Ohkawara et al. (32) measured fructosamine levels using extracted albumin and a fructosamine assay that depends on the potential of the glycated proteins to reduce nitroblue tetrazolium. In that study, the corrected albumin fructosamine values correlated more closely with fasting blood glucose levels (r = 0.735) than the serum fructosamine values corrected for albumin (r = 0.514; ref. 32). Although the correlation coefficient we observed in our study between serum glucose and fructosamine were significant, they were considerably lower compared with the study by Ohkawara and we did not account for serum albumin levels.

Another concern is the biological and technical variation of fructosamine (18). For instance, previous reports indicated that samples could be stored for several months at −20°C, but large changes have been observed in frozen samples (18). Prolonged storage at ultra-low temperatures (−196°C) prevents *in vitro* glycation of serum proteins (18). Balland et al. (33) examined storage effects on human serum fructosamine concentration at 1 hour, 1 week, and at 6 months. These samples were stable over 6 months at −40°C and at −196°C (33). They concluded, however, that samples should not be kept longer than 1 week at +40°C, but could be kept for 6 months at −196°C (34). In our study, the samples were stored at −80°C until biochemical determination. However, case-control sets were matched on time since recruitment.

In addition to technical variation, other factors may contribute to variability in fructosamine levels. Some of these physiologic variables include prolonged bed rest, strenuous exercise, circadian variation, and diet (29). We did control for circadian differences as blood was collected at a specific time of day for all participants and all levels were fasting, but we were unable to control for the other factors. However, when we looked at serum fructosamine levels among all women, there was a significant difference between current smokers and nonsmokers, but the addition of smoking status, number of cigarettes smoked per day or peak number of cigarettes smoked did not change fructosamine risks when added to the regression model.

Premenopausal and postmenopausal women differed in fructosamine levels. This difference may be a reflection of differences in glucose levels we observed between these two groups of women. In addition, although levels were fasting, serum fructosamine levels will reflect intake from the past several weeks. As such, the level reflects usual dietary intake and not necessarily current intake. This could explain the differences in correlation between premenopausal and postmenopausal groups. Perhaps, postmenopausal women have a less varied diet and less physical activity than premenopausal women and thus the fructosamine levels of postmenopausal women are more consistent with current glucose levels.

Table 2. Adjusted risks (odds ratios) and 95% CI by serum levels of fructosamine

Fructosamine level	Cases/controls	Overall odds ratios* (CI)
≤1.76†	26/129	1.00
>1.76 to ≤1.88	50/201	1.19 (0.71-2.05)
>1.88	55/174	1.60 (0.95-2.73)

*Adjusted for age, age at menarche, age at first birth, parity, and BMI.
†Reference category.

In conclusion, we found that serum fructosamine, as an indicator of glucose consumption, may be a predictor of breast cancer. The 60% increase in risk was similar for both premenopausal and postmenopausal women but the results failed to reach statistical significance. Nevertheless, further investigation of this biomarker of glucose metabolism is of interest as a potential additional mechanism explaining breast cancer etiology.

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The Role of Endogenous Hormones in the Etiology and Prevention of Breast Cancer: The Epidemiological Evidence

Paola Muti (✉)

P. Muti
Department of Social and Preventive Medicine, University at Buffalo, State University of New York, 270 Farber Hall, 3435 Main Street, Buffalo, NY 14214, USA

✉ P. Muti
E-mail: muti@buffalo.edu

Abstract Breast cancer is the most common cause of cancer death in women worldwide. Rates vary about fivefold around the world, but they are increasing in regions that until recently had low rates of disease. Despite the numerous uncertainties surrounding the etiology of breast cancer, intensive epidemiological, clinical, and genetic studies have identified a number of biological and social traits as risk factors associated with breast cancer. Principal among them is the evidence of BRCA1 and BRCA2 susceptibility genes, familial history of breast cancer, age, higher socioeconomic status, ionizing radiation, tallness in adult life, alcohol consumption, and a variety of hormone and metabolic factors. Among the hormonal influences, a relevant etiological function has been ascribed to unopposed exposure to elevated levels of estrogens and androgens. In addition, new epidemiologic evidence has indicated that among the metabolic factors, glucose metabolism, hyperinsulinemic insulin resistance, and insulin-like growth factor bioavailability may also play a role in breast cancer. These endocrine and metabolic factors may represent future targets for breast cancer prevention.

Breast cancer is the most common cause of cancer death in women worldwide. Rates vary about fivefold around the world, but they are increasing in regions that until recently had low rates of disease [1–3]. Despite the numerous uncertainties surrounding the etiology of breast cancer, intensive epidemiological, clinical, and genetic studies have identified a number of biological and social traits as risk factors associated with breast cancer. Principal among them is the evidence of BRCA1 and BRCA2 susceptibility genes, familial history of breast cancer, age, higher socioeconomic status, ionizing radiation, tallness in adult life, alcohol consumption, and a variety of hormone and metabolic factors [4, 5]. Among the hormonal influences, a relevant etiological function has been ascribed to unopposed exposure to elevated levels of estrogens and androgens [4–7]. In addition, new epidemiological evidence has indicated that among the metabolic factors, glucose metabolism, hyperinsulinemic insulin resistance, and insulin-like growth factor bioavailability may also play a role in breast cancer. These endocrine and metabolic factors may represent future targets for breast cancer prevention.

1 Introduction

In 1896, Beatson was the first to hypothesize the influence of ovarian activity on formation and progression of breast cancer [8]. At that time, these hormones were not known to be a unique class of substances. The first experimental proof of their presence in follicular liquids of a premenopausal ovary and their cancer promotion potential was shown more than 30 years later by Lacassagne [9]. In vitro and in vivo studies using natural, synthetic, or both kinds of sex steroid hormones demonstrated their potential in the formation and progression of benign and malignant tumors [10–11].

Epidemiological evidence of an association between sex steroid hormones and breast cancer risk based on retrospective study design, such as case-control studies, has been generally inconsistent. When the results were consistent across a few independent studies and supportive of the association of hormones and breast cancer, the findings were still compatible with the non-causal hypothesis that high hormone levels in breast cancer cases were due entirely or in part by the presence of the tumors or as consequence of the disease. Because of the disease-status effect on the endocrine or metabolic profile, this report describes only evidence from prospective cohort studies.

2 Sex Hormones and Breast Cancer in Postmenopausal Women

The hypothesis that cumulative exposure of breast tissue to ovarian hormones is one of the major determinants of breast cancer has existed for at least 30 years. Epidemiological evidence has been well corroborated the existence of the association in postmenopausal women. During the last 10 years, nine research groups have published results from prospective studies of endogenous hormones and breast cancer: Columbia, MO, USA [13, 14]; Guernsey, UK [15]; Nurses' Health Study, USA [16]; New York University Women's Health Study (NYU WHS), USA [17, 18]; Study of Hormones and Diet in the Etiology of Breast Tumors (ORDET), Italy [19]; Rancho Bernardo, USA [20, 21]; Radiation Effects Research Foundation (RERF), Japan [22]; Study of Osteoporotic Fractures (SOF), USA [23]; and Washington County, USA [24, 25]. These studies, based on recruitment of thousands of healthy women and on their epidemiological surveillance, have indicated that high levels of estrogens and androgens precede the occurrence of breast cancer risk in postmenopausal women.

A recent pooled analysis of these nine large prospective cohort studies has then further supported the role of endogenous hormones in the etiology of breast cancer [26].

As reported in Fig. 1, in the pooled analysis of the prospective studies examining risk by quintiles of hormone serum concentration, both estrogens and androgens were significantly associated with an increase in breast cancer risk, with evidence of a dose-response relationship. The relative risk for breast cancer for women in the highest quintile for estradiol compared with women in the lowest quintile was 2.00 (95% confidence interval 1.47–2.71). The relative risks in the highest quintile compared with the lowest quintile for the other estrogens and the androgens were all approximately 2, and the highest relative risks were in the highest quintiles for free estradiol [relative risk 2.58 (1.76–3.78)] and non-sex hormone binding globulin (non-SHBG)-bound estradiol [relative risk 2.39 (1.62–3.54)]. For SHBG there was a significant inverse association with breast cancer risk [relative risk in top fifth 0.66 (0.43–1.00)].

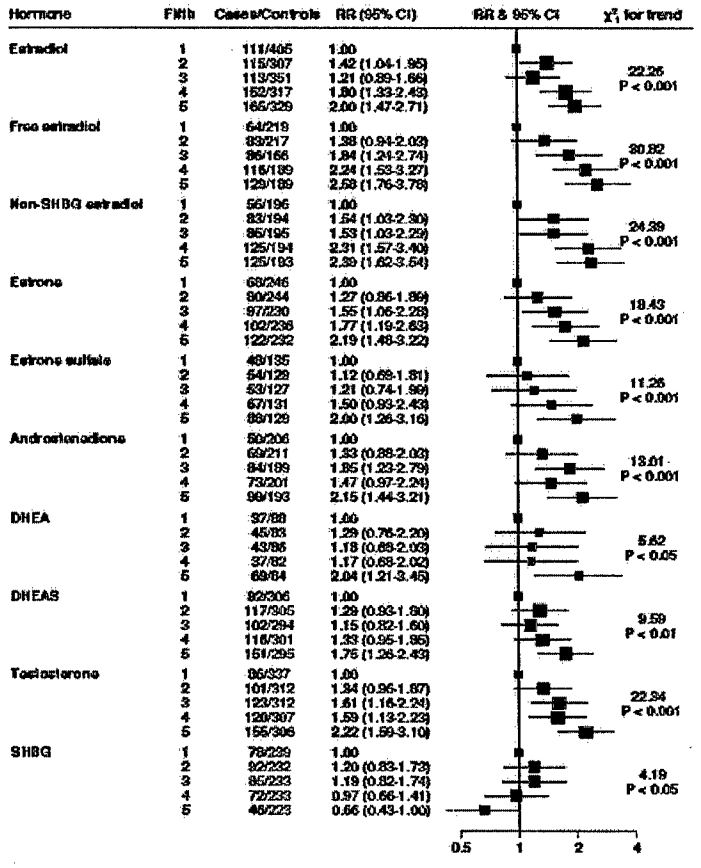

Fig. 1. Relative risk (*RR*) of breast cancer by fifth of hormone concentration. *CI* confidence interval, *DHEA* dehydroepiandrosterone, *DHEAS* dehydroepiandrosterone sulfate, *SHBG* sex hormone binding globulin. (From [26])

Although the postmenopausal ovaries secrete a very small amount of estrogens, circulating estrogens in women after menopause are still produced through peripheral aromatization of the androgens, primarily androstenedione and testosterone. Thus, part of the etiological relation linking serum androgens to breast cancer could be explained by their aromatization into estrogens. In the pooled analysis, we separated by adjustment and stratification the effect of androgens on breast cancer risk from the effect of estrogens. We observed that the association between androgens and breast cancer held after adjustment for estrogens, indicating an independent effect of androgens on breast cancer risk.

Thus, results of this pooled analysis of the worldwide data from prospective studies has established not only that serum concentrations of endogenous sex hormones are precursors of breast cancer in postmenopausal women, but also that both estrogens and androgens are independently associated with the development of the disease through two possible independent pathways. While circulating estrogens may act directly on the breast tissue and breast cancer cells,

the action of serum androgens may be mediated through their aromatization into estrogens within breast tissue and in breast cancer cells [27].

3 Sex Hormones and Breast Cancer in Premenopausal Women

The normal human ovaries produce all three classes of sex steroids: estrogens, progesterone, and androgens and all three have been considered in analytical studies on breast cancer etiology in premenopausal women.

Among the hormonal influences, a major role has been attributed to the unopposed exposure to elevated levels of estrogens. Various analytical studies on estrogens and breast cancer risk led to contradictory results irrespective of the type of estrogens they were analyzing [28]. Estradiol is by far the most potent and the highest concentrated naturally occurring estrogen in premenopausal women. Thus, epidemiological studies conducted in premenopausal women have usually focused on estradiol in their analysis. Prospective studies with information from premenopausal women reported higher follicular but lower luteal estradiol in premenopausal women who subsequently developed breast cancer than in a sample of cohort members of the Washington County prospective study chosen as controls [25]. The opposite was previously found by Wysowski et al. [29], in the same cohort study. Rosenberg et al. [30] reported, in a case-control study nested in the New York University Women's Health Study, similar estradiol levels in cases and controls (although further adjustments for stage of menstrual cycle at blood drawing suggested that estradiol was on average non-significantly higher in cases). Kabuto et al. [22] found in the prospective cohort study conducted in Japan higher levels of bioavailable estradiol in breast cancer cases than in controls. Results from the prospective study conducted in the island of Guernsey (UK) by Key and colleagues showed that premenopausal breast cancer cases excreted less estrogen than controls when estrogens were determined in urine [31] and that estrogen levels were higher in cases than in controls when the hormones were determined in blood, although the difference was small and not statistically significant [32]. The number of breast cancer cases in those studies ranged between 22 [25] and 79 [30]. Several of those studies tried to control the ovarian phase variability using time interval between the date at specimen collection and the date at the subsequent menstrual period either as a matching variable or variable to adjust for in the analysis [30–32]. On the contrary, Wysowski et al. [29] and Helzlsouer et al. [25] used the time interval between date at the menstrual period preceding the blood collection and the date at blood drawing, while Kabuto et al. [22] did not

control for menstrual phase. Only Helzlsouer et al. [25] controlled for hormone circadian rhythm, matching the set of cases and controls on time-of-the-day at blood drawing, although no specification on this matching criterion was given. All determinations performed in blood used radioimmunoassay methods, although none specified whether the determinations were done using direct or indirect methods and single or duplicate assays. This information may have influenced the technical variability of the hormone determination and thus the precision of the observed risk estimates.

Almost all prospective studies analyzing the relation of breast cancer with endogenous androgens in premenopausal women showed a positive association of testosterone levels with risk, with the only exception of Wysowski et al.[29] who did not find a difference in testosterone levels between breast cancer cases and controls in his nested case-control study. However, all observed risks were of low magnitude and not statistically significant [22, 25, 32].

During the menstrual cycle, progesterone, in conjunction with estrogens, regulates the functions of the sex organs. This hormone is important in preparing the uterus for implantation of the blastocyst and in maintaining pregnancy. In nonpregnant women, progesterone is secreted mainly during the luteal phase of the ovarian cycle by the corpus luteum, a yellow glandular mass in the ovary formed by an ovarian follicle following the discharge of its ovum.

Only a few prospective cohort studies have reported the association of luteal phase progesterone levels with subsequent breast cancer, but the number of cases was very small. Thomas et al. [32] reported a 9% lower mean serum concentration of progesterone, measured in early luteal phase, in cases than in controls (the study was based on 12 breast cancer cases). Wysowski et al. [29] found a 29% lower mean concentration of progesterone in cases than in control subjects after matching on time since last menstrual period (based on 17 breast cancer cases). Helzlsouer et al. [25], contrarily, reported a higher concentration of luteal phase progesterone in cases, but the study was based on nine breast cancer cases only. None of these differences was statistically significant.

In summary, evidence derived from prospective cohort studies is consistent to some extent, at least for the association of androgens with breast cancer. However, the small number of breast cancer cases in these studies and the difficulty in controlling hormone variability over the ovarian cycle may have weakened the strength of the observed association.

4 Hyperinsulinemic Insulin Resistance, Insulin-Growth Factor Bioavailability, Glucose Metabolism, and Breast Cancer Risk

In addition to the sex steroid hormones, there is some reason to believe that insulin and insulin-like growth hormone (IGF)-I and glucose metabolism may also play a role in breast cancer etiology.

Insulin is a powerful mitogenic agent [35], inducing a dose-dependent growth response in breast cancer cell lines acting via insulin receptor [36]. Moreover, insulin may also play a role in tumor promotion by up-regulation of ovarian steroid secretion [37]. Overall, insulin stimulates androgen production in ovarian tissue samples in in vitro studies [38–40].

IGF-I is a small peptide (about 7,500 Da) with a significant structural homology with proinsulin and insulin [41], which is highly regulated by growth hormone (GH) [42]. Despite their distinct immunological difference, IGFs and insulin share not only important similarities in their structure, their receptors, and their signaling pathways which determine their biological actions, but they also have a common ancestor, possibly an old serine protease [43]. The ancestor molecule may have stimulated cell and tissue growth after food intake, and this function probably included some "insulin-like activity." The latter seems to have been refined by the emergence of proinsulin, whereas growth-promoting activity has been preserved mostly in the IGFs. Thus, despite the divergence of their biological functions and their refinement and adaptation to specific purposes, both insulin and IGFs share some common functions: IGFs respond to hyperglycemic stimulus and exert acute effects on metabolism, and insulin is able to stimulate growth [44, 45]. IGF-I stimulates multiple cellular responses that are related to growth, including synthesis of DNA, RNA, and cellular proteins [46]. IGF-I has well-documented effects on cell proliferation, and similarly to insulin, IGF-I has been shown to inhibit programmed cell death (apoptosis) [42–49]. Furthermore, in breast cancer cell lines, concentrations of insulin and IGF-I receptors are increased [50, 51]. The biological activity of IGF-I within tissues, including breast epithelium, is regulated by a family of major plasmatic binding proteins (IGFBPs), and partially also by the local production of IGF-I and IGFBPs within tissues [42, 52–53]. At least seven different IGFBPs have been identified so far, but only three of these (IGFBP-1, -2, and -3) are found at significant levels in blood. Over 90% of IGF-I is bound with IGFBP-3 plus another glycoprotein, called acid-labile subunit (ALS). Most of the remaining fraction is bound to the smaller binding proteins IGFBP-1

and IGFBP-2. A decrease in plasma IGFBP-3, with a transfer of IGF-I to IGFBP-1 or IGFBP-2, may result in greater IGF-I availability to its tissue receptors, since the large IGF-I/IGFBP-3/ALS complex cannot pass through the capillary barrier to target tissues, while the smaller complexes of IGF-I with IGFBP-1 or IGFBP-2 can [42, 53].

There is increasing evidence that IGF-I is also a direct modulator of the formation and biological availability of ovarian steroid hormones. IGF-I has been shown to share with insulin the function to up-regulate the secretion of sex steroid hormones and increase their bioactivity through the inhibition of sex hormone-binding globulin secretion in the liver [54–56].

There is consistent prospective epidemiological evidence of a close association between IGF-I and breast cancer risk, however more often in premenopausal women [57–60]. To date, three prospective studies have been conducted on serum insulin or C-peptide and breast cancer risk [58, 59, 61]. No evidence for a positive association between C-peptide and breast cancer was found by Jernström et al. in older postmenopausal women [61]; however, the study was limited by the small sample of breast cancer cases included in the analysis (45 breast cancer cases). Toniolo et al. [58] reported a positive association of C-peptide with premenopausal and postmenopausal breast cancer risk that was not statistically significant. Nonfasting condition at blood collection for these studies may, at least in part, explain the weakness of the observed association. In our recently published analysis [59], using a nested case-control study in the ORDET cohort prospective cohort, we observed a 70% relative risk increase for breast cancer in the two highest quartiles of fasting insulin levels; however, all the confidence intervals included unity.

Glucose may play a direct role in the development of breast cancer by favoring the "selection" of malignant cell clones [62]. Neoplastic cells have been shown to extensively utilize glucose for proliferation [62]. Increased metabolism of glucose toward the pentose phosphate pathways is one of the central metabolic characteristics of malignant tissues [62].

In our above-mentioned study [59], we also analyzed the hypothesis that serum fasting glucose is associated with breast cancer. In premenopausal women, glucose was strongly and significantly associated with breast cancer risk: the age, body mass index (BMI), and reproductive variable adjusted relative risk for the highest quartile of serum glucose versus the lowest was 2.8 [95% confidence interval 1.2–6.5], p=0.02.

5 Conclusions

Breast cancer incidence rates are higher in Western countries than in Africa or Asia. Although both genetic and environmental factors may explain the large geographic variation in incidence rates, studies on migrants who moved from countries characterized by low incidence (i.e., Japan) to countries with higher incidence (i.e., the United States and Italy) showed a significant increase in breast cancer incidence in individuals that migrate in comparison with their peers in the countries of origin. This evidence suggests that environmental factors play a significant role in breast cancer development. In countries with high breast cancer incidence rates, lifestyle is characterized by an energy-dense diet rich in total and saturated fat and refined carbohydrates, and by low physical activity. A sedentary life and a high-fat, low-complex-carbohydrate diet have been associated with impaired glucose metabolism, hyperinsulinemic insulin resistance, and elevated serum levels of androgens and estrogens, the metabolic and endocrine patterns previously described to be associated to breast cancer risk. Hormones and metabolic factors therefore, might represent a possible etiological linkage between lifestyle characteristics and breast cancer.

Recent studies have observed the efficacy of changes in diet and in lifestyle in improving insulin sensitivity and reducing the availability of sex hormones [63–73]. These studies may indicate possible strategies for future breast cancer prevention.

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