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INCREASED HOMOCYSTEINE PLASMA LEVELS IN BREAST CANCER PATIENTS OF A MEXICAN POPULATION

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***Aim*: Hyperhomocysteinemia has been associated with different pathologies, including cardiovascular diseases, hypertension, diabetes, and breast cancer (BC). To examine the differences in total homocysteine (tHcy) plasma levels, we compared healthy women to BC patients from a Mexican population. *Materials and Methods*: The tHcy plasma levels were measured using high-performance liquid chromatography with a fluorescence detector in 89 female controls and 261 BC patients. *Results*: The observed plasma tHcy levels were significantly higher among the BC patients (11.1019 ± 5.9161 μmol/l) compared to the controls (9.1046 ± 1.3213 μmol/l) ($p = 0.002$), and these differences were evident when stratified by age (⩾ 50 years old), menopause status, overweight and obesity, miscarriages, node metastases, progression, subtype classification (luminal, Her2 and triple negative) and nonresponse to chemotherapy. *Conclusions*: The tHcy plasma levels could be a good marker for the progression and chemosensitivity of BC in the analyzed sample from a Mexican population.**

***Key Words:* plasma levels, homocysteine, HPLC, breast cancer, Mexican population.**

Breast cancer (BC) is a significant public health problem worldwide [1], and its incidence varies among different ethnic groups [2–4]. In Mexico, BC is responsible for 19% of mortality in women > 20 years old and in general is considered one of the major causes of death [4]. A study that projected cancer incidence to 2030 in Mexico estimated that there will be approximately 13,981 new cases annually, of which 38% will occur in women older than 65 years [4]. BC develops through a gradual accumulation of genetic and epigenetic changes that transform normal breast cells into invasive cancer tissues [5]. In this sense, previous studies have reported the relationship between hyperhomocysteinemia (HHcy) and BC [6–8].

Homocysteine (Hcy) is a sulfur amino acid precursor of the demethylation of methionine, and it is degraded by remethylation or transsulfuration [7]. Vitamins B_6 and B_{12} and folate are important cofactors that participate in the metabolism of Hcy [9]. Hcy is present as a monomer or a dimer (Hcy-Hcy, Hcy-cysteine) and is mainly bound to a protein fraction (albumin; 80–85%) [10]. Hcy also plays an important role in methylation and the provision of nucleotides for DNA synthesis. The insufficient input of folates produces elevated plasma concentrations of Hcy and S-adenosylmethionine (SAM); thus, SAM acts as an inhibitor of the methyltransferase enzyme. This inhibition alters both the DNA methylation process and the regulation of gene expression, and consequently participates in the oncogenesis on the genetic level [11].

Conversely, different factors can affect Hcy levels such as age, gender, menopause, pregnancy, tobacco and alcohol consumption, ethnic group and deficiency of enzymes involved in the methionine to cysteine metabolism. The reference ranges of Hcy, in healthy individuals, are determined by standardization in each laboratory [12, 13]. Some studies have associated HHcy with BC [8] with low folate levels [14], metastasis [15], and chemotherapy response [16]. However, in the Mexican population, these associations remain unknown. Thus, the aim of this investigation was to determine the HHcy plasma levels in Mexican BC patients.

MATERIALS AND METHODS

Blood samples were collected from 89 healthy female blood donor volunteers and from 261 patients with clinically and histologically confirmed BC. All patients were residents of the metropolitan area of Guadalajara and were recruited from June 2014 to May 2017. All samples were obtained after

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Abbreviations used: BC – breast cancer; BMI – body mass index; Hcy – homocysteine; HHcy – hyperhomocysteinemia; HPLC – high-performance liquid chromatography; SAM – S-adenosylmethionine; SAH – S-adenosylhomocysteine; tHcy – total homocysteine.

the patients signed a written informed consent, previously approved by the ethics committee (IMSS 1305). All procedures performed in studies involving human participants were in accordance with the 1964 Declaration of Helsinki. All included individuals were unrelated. Clinical and demographic data were obtained using written questionnaires. All patients were interviewed to determine their occupational exposure and current drug regimens.

tHcy quantification was performed by the HPLC-fluorescence method (Agilent 1100 series HPLC system equipped with an isocratic pump, thermostat, automatic injector, model 1100 fluorometric detector system controlled by the ChemStation software), according to a modified method described previously [17], using 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F, Sigma-Aldrich, St. Louis, MO, USA) as the derivatizing agent.

The plasma samples were separated by centrifugation (3,500 rpm × 10 min) at room temperature within 30 min after collection and stored at −20 °C until being processed. To separate tHcy, 50 µl of plasma sample, 25 µl of 1X PBS (phosphate buffered saline) and 40 µl of Tris (2-carboxyethyl) phosphine hydrochloride 25 g/l (TCEP, Sigma-Aldrich, St. Louis, MO, USA) were incubated for 30 min at room temperature in an Eppendorf tube for thiol reduction. Subsequently, 90 µl of trichloroacetic acid-ethylenediaminetetraacetic acid (TCA-EDTA 0.6 M and 0.0012 M, respectively) was added and mix for 30 s for proteins precipitation. After centrifugation at 13,000 g for 10 min at room temperature, from the supernatant 50 µl were taken, and then 40 µl of 0.39 M NaOH, 125 µl of borate-EDTA buffer (0.005 M EDTA and 0.125 M boric acid) and 50 µl of g/l SBD-F derivatizing agent were added. The mixture was homogenized and incubated for 60 min at 60 °C in a light-protected water bath. Filter sample by 0.45 µm Millipore filter and injected into the chromatograph.

Acetate buffer (mixture of 0.083 M sodium acetate and 0.05 M acetic acid, adjusting the pH to 5.5 with NaOH) was used as the mobile phase in an Eclipse C18 column (150 × 4.6 mm, 5 µm). The fluorometric detector had a length (λex) of 385 nm for excitation and 515 nm for emission. The validation process was conducted following the guidelines for biological sample analyses [18].

All statistical analyses were performed using the PASW Statistic Base 18 software, 2009 (Chicago, IL, USA). Sample *t*-tests or the Mann — Whitney U test were performed to compare the means of a continuous variable across dichotomized variables. All statistical tests were two-tailed, and *p* values less than 0.05 were considered statistically significant.

RESULTS

Table 1 shows the comparative epidemiological data from the BC patients and the control individuals. The observed average age was 53.3 ± 11.4 years in the patient group and 52.6 ± 15.5 years in the control group; non-significant differences were observed. However, the tHcy plasma levels were significantly different between the patients and controls ($p = 0.002$), and they remained significantly different when stratified by ⩾ 50 years old ($p = 0.015$) and menopause status ($p = 0.007$).

Table 1. Comparison of Hcy plasma levels in BC patients and controls

Characteristics	BC patients	Controls	*p*-value*
	tHcy, µmol/l (mean ± SD)		
Age, years	53.3065 ± 11.4427 (n = 261)	52.6180 ± 15.4636 (n = 89)	0.656
tHcy	11.1019 ± 5.9161 (n = 261)	9.1046 ± 1.3213 (n = 89)	**0.002**
tHcy (< 50 years old)	10.4886 ± 4.4830 (n = 89)	9.0317 ± 1.4298 (n = 38)	0.053
tHcy (⩾ 50 years old)	11.4193 ± 6.5251 (n = 172)	9.1590 ± 1.2461 (n = 51)	**0.015**
tHcy (tobacco consumption)	10.4485 ± 3.2310 (n = 72)	9.2481 ± 1.5462 (n = 19)	0.120
tHcy (alcohol consumption)	10.0792 ± 3.3334 (n = 38)	9.0777± 1.4435 (n = 18)	0.229
tHcy (menopause)	11.4408 ± 6.5051 (n = 173)	9.1014± 1.2387 (n = 59)	**0.007**
tHcy (premenopause)	10.4358 ± 4.5012 (n = 88)	9.1109± 1.4929 (n = 30)	0.117

Notes: SD – standard deviation; *Student's *t*-test.

When stratified and compared by menopause and pre-menopause status and alcohol and tobacco consumption, there were no significant differences between the patients and the controls (Table 1). Significant differences were found in tHcy plasma levels between BC patients, stratified by clinical characteristics, compared to the control group (Table 2). Non-significant differences in tHcy plasma levels were found between BC groups stratified by clinical characteristics.

DISCUSSION

In Mexico, BC is currently one of the leading causes of disease and death in women [1–4]. In the present study, we observed that the mean age of the BC patients was 53.3 ± 11.4 years. Many studies have observed a high incidence of BC in patients who were approximately 50 years old [1–5, 19].

Lifestyle changes and expanded longevity likely have an influence on the increased frequency of this disease in the Mexican population [3, 19, 20].

Knowledge of how Hcy metabolism affects breast molecular carcinogenesis is important; relevant studies have reported that HHcy in women with low folate status affects gene expression and promotes cell proliferation in the oncogenesis of breast cells [5–10, 14–16, 21]. However, the relationship between HHcy and BC remains controversial and possibly depends on the population being studied and ambient factors including lifestyles and food and toxic exposition that can influence the promotion of tumoral epigenetic changes [15, 22].

Moreover, little is known regarding tHcy levels in Mexican BC patients. In our study group, the Hcy levels (mean ± SD) were 9.1046 ± 1.3213 µmol/l in controls and 11.1019 ± 5.9161 µmol/l in BC patients, which indicates the presence of HHcy in BC ($p = 0.002$); these data are consistent with other studies in BC [8, 15, 16, 21]. Furthermore, the tHcy levels in women ⩾ 50 years

old and with menopause status differed between the control group and the BC patients ($p = 0.015$).

Some studies suggest that HHcy is related to individual differences such as gender, diet, alcohol consumption and smoking habits [15, 21, 23]. Others established that HHcy was more evident in older subjects than in younger subjects [24]. In fact, the HHcy in BC patients ⩾ 50 years old observed in this study could be due to the average age of our BC patients and may also be influenced by hormonal effects, which can modulate plasma Hcy [24, 25].

Hcy metabolism may regulate the expression of distinct pathways required in tumoral behavior, which has been demonstrated in previous studies in BC [14, 21]. A hypothesis by Zhu *et al.* [26] proposes that HHcy is a risk factor for estrogen induced tumorigenesis: HHcy causes the intracellular accumulation of S-adenosylhomocysteine (SAH), which induces the production of oxidative metabolites of estrogens (catechol estrogens) that contribute to estrogen-induced tumors in animal models and in some human cancers such as BC [7].

Nevertheless, HHcy is considered a risk factor in BC patients with increased body mass index (BMI; overweight, obesity). A variety of mechanisms for obesity and HHcy have been proposed that could contribute to the development of BC [27].

In addition to these mechanisms, several factors could influence the development of BC. As a previous study has suggested, different dietary patterns may contribute to the observed differences in Hcy, which could upset the balance between the production and degradation of Hcy, resulting in HHcy [27]. These results suggest that the dietary pattern of studied population is rich in fat, food processing and freezing, which could contribute to weight gain in women with BC. In fact, there are several theories that attempt to explain this association, involving the role of leptin, insulin and other molecules that mediate the inflammatory process, independent of estrogen and HHcy [20]. Hcy may inhibit endothelial cell growth, increasing oxidative stress and promoting inflammatory processes in adipocytes, which could collectively accelerate oncogenesis in BC [28]. Adipocytes are now recognized as active endocrine cells that produce hormones, growth factors, and cytokines. In fact, the most likely scenario is that these mechanisms may act in combination to explain the relationship linking menopause and subsequent weight gain as a risk factor for BC progression and mortality [20]. It is well established that folate deficiency results in HHcy. Hence, in folate-deficient individuals, decreased levels of tetrahydrofolate (THF) cofactors (e.g., 5-CH_3-THF) limit metabolic flux through the methionine synthase reaction, with the consequent accumulation of Hcy, the substrate of this enzyme [29].

Samples analyzed in this study showed that the factors associated with HHcy were age (⩾ 50 years old), menopause and BMI (overweight and obesity), factors that are risk indicators for different pathologies including BC (Tables 1 and 2). These factors are closely related since estrogen production is lower in older women, which consequently results in overweight or obesity, and obesity is related to menopause because of a greater endogenous estrogen production. This, in turn, is related to the altered metabolism of Hcy. Thus, estrogens stimulate the metabolism of methionine by transamination and in turn increase the catabolism of Hcy [30].

The association of HHcy with miscarriages in BC patients was evident in the present study. This relationship is not well established in previously studies; however, some studies have observed HHcy in recurrent abortions [31]. HHcy may be consequence of an interaction between a genetic defect (presence of mutations in enzymes that participate in folate

Table 2. Comparison of Hcy plasma levels by clinical characteristics of BC patients and controls

Clinical characteristics		tHcy, μmol/l (mean ± SD)		
		BC patients	Controls (n = 89)	*p*-value*
BIM**	Overweight (18.5–24.9 kg/m^2) (n = 86)	10.7126 ± 5.0415	9.1046 ± 1.3213	0.002
	Obesity I (25–29.9 kg/m^2) (n = 69)	12.1896 ± 9.2362		0.001
	Obesity II (30–34.9 kg/m^2) (n = 27)	11.1674 ± 4.0041		0.001
	Obesity III (35–39.9 kg/m^2) (n = 9)	11.0708 ± 1.6192		0.001
	Miscarriages (n = 86)***	10.7473 ± 3.7162		0.001
	Node metastasis (n = 183)	11.5412 ± 6.7426		0.001
Stage	I (n = 14)	10.0079 ± 3.1161		0.062
	II (n = 76)	10.8486 ± 8.1610		0.049
	III (n = 81)	10.9711 ± 4.8267		0.001
	IV (n = 86)	11.4978 ± 4.7680		0.001
HC	Triple negative (n = 89)	10.0293 ± 3.6542		0.033
	Luminal A (n = 114)	11.4753 ± 7.8658		0.005
	Luminal B (n = 56)	11.6637 ± 4.3413		0.001
	HER2 (n = 42)	10.5910 ± 2.8378		0.001
	Ki-67 (n = 173)	10.7807 ± 6.1952		0.012
Chemotherapy	Before (n = 47)	14.0525 ± 10.3094		0.001
	After (n = 214)	10.4539 ± 4.17458		0.003
	NR (n = 13) ****	14.1158 ± 8.76301		0.001
	MR (n = 38)	10.7665 ± 3.35320		0.001
	NRR (n = 47)	11.3949 ± 4.79354		0.001

Notes: SD – standard deviation; HC – histologic classification; *Student's *t*-test; **according to OMS classifications (appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Genève (Switzerland): World Health Organization, 2004); NRR – nonresponse chemotherapy by recurrence; ***⩾ 2 loss gestational; ****NR – non-chemotherapy response; MR – minimal chemotherapy response; NRR – non-chemotherapy response by recurrence. The nonresponse to treatment with (epirubicin-cyclophosphamide-5-fluorouracil) and capecitabine was evaluated according to the pathological Ryan's classification described as follows: 1. Moderate response (single cells or small groups of cancerous cells); 2. Minimum response (residual cancer surrounded by fibrosis); and 3. Poor response (minimal or no tumor destruction, extensive residual cancer).

metabolism), a nutrition condition (folate deficiency), and preeclampsia, which could be a putative cause of recurrent miscarriages [31].

HHcy and node metastasis, Ki-67 and advanced cancer stages (III and IV) were also statistically significant in our BC patients. A variety of HHcy mechanisms could contribute to the development of metastasis. In fact, a significant correlation has been observed between the number of Ki-67 positive cells and tumor cell proliferation [32]. Other authors have suggested that the activation and proliferation of immunocompetent cells are responsible for HHcy in BC and thromboembolic events in patients with malignancies [33]. Moreover, a strong staining of folate receptor alpha in Stage IV BC has been observed, suggesting the necessity of folates demands in advanced tumoral progression [34, 35].

We observed the presence of HHcy in luminal (A and B), HER2 and triple negative cases. In addition, there are other studies that support our findings [35]. HHcy was observed in BC patients, with no response to adjuvant chemotherapy, contained such chemotherapeutic drugs as capecitabine (oral drug) and epirubicin-cyclophosphamide-5-fluorouracil (prodrug) that are commonly given to BC patients. The alkylating agents of chemotherapy would affect folic acid and B_{12} vitamin concentrations, resulting in increased Hcy concentrations, which act on DNA by affecting synthesis and cell division. Folic acid antagonists, such as capecitabine and 5-fluorouracil, inhibit both dihydrofolate reductase and folate-dependent enzymes in the synthesis of nucleic acids, consequently HHcy alter the pharmacological efficiency of chemotherapeutic agents interfering their action mechanism, which affects the response in BC. Furthermore, some neoplastic cell receptors have an affinity to folates; thus, they are dependent on the folate concentration, and HHcy can cause a lack of sufficient supplies. The chemosensitivity of patients with BC differs from person to person, which reflects the limited advances in our understanding of the molecular mechanisms underlying breast carcinogenesis and the individual susceptibility to this disease and its treatment [19].

Folate metabolism can likely influence the chemotherapy response in addition to other factors, such as a known presence of metastatic nodes, tumor markers, menopause, the time of diagnosis, tumor stage, and treatment resistance [19]. This response might be affected by polymorphisms in genes that participate in folate metabolism, which could lead to a dysregulation in the metabolite concentration, thus producing changes in DNA and subsequently participating in neoplastic progression. Aberrant DNA methylation is recognized as a common feature of human neoplasia, with CpG island hypermethylation and global genomic hypomethylation occurring simultaneously in tumors. Because it affects the methylation of DNA and tumor suppressor genes, the *MTHFR* polymorphism could potentially modulate the efficacy of cytotoxic agents [20, 21, 25]. Moreover, the response to drugs is not only related to the monogenic inheritance of a protein but also depends on genes encoding proteins involved in multiple metabolic pathways, posttranslational modifications, gene interactions, and epigenetic regulation [21].

Unfortunately, the status of folate, vitamin B and dietary intake of the individuals were not analyzed in this study. Nonetheless, the results suggest that an appropriate dietary folates intake can reduce BC risk in older women, which could be reflected in progression and susceptibility to chemotherapy.

In conclusion, our results showed that when comparing HHcy levels in BC patients and controls, the differences were more evident with the following characteristics: 1) $\geqslant$ 50 years old; 2) menopause; 3) BMI overweight and obesity; 4) miscarriages; 5) node metastases; 6) progression; 7) luminal, HER2 and triple negative; and 8) nonresponse to chemotherapy, confirming that tHcy plasma levels could be a good marker of BC and that these factors contribute significantly to BC in the analyzed sample from a Mexican population. Further studies are required to confirm these observations.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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