

Aim of the study: The aim of this pilot study was to evaluate the plasma total antioxidant capacity (TAS) in breast cancer patients in relation to ER β expression.

Material and methods: The study group consisted of newly diagnosed consecutive female breast cancer patients ($n = 41$) and controls ($n = 28$) randomly selected from women with benign breast disease. TAS was determined with the ABTS reagent. Immunostaining for ER β was performed using polyclonal antibodies. ER α , PgR and HER-2 were measured routinely (immunostaining for ER α and PgR with monoclonal antibodies and EnVision detection system; immunohistochemical method/FISH for HER-2 expression).

Results: The plasma TAS was significantly decreased in the breast cancer patients in comparison to the controls independently of hormonal and lymph node status. The TAS level was not significantly different between breast cancer subgroups either in relation to the ER β expression (ER β + vs. ER β -) or considering the steroid receptor status (ER α +, ER β +, Pg+ vs. ER α +, ER β -, Pg-) even in the selected lymph node negative subgroup. Similarly, HER-2 expression did not significantly affect the TAS concentration. A tendency towards higher TAS level in all ER β negative breast cancer subgroups was observed.

Conclusions: The results might confirm enhanced consumption of plasma antioxidants in breast cancer patients. The determination of ER β isoforms along with parameters of redox status might enable better understanding of their mutual influence.

Key words: total antioxidant status, oxidative stress, breast cancer, estrogen receptor, estrogen receptor β .

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Total antioxidant status in plasma of breast cancer patients in relation to ER β expression

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Introduction

Prolonged lifetime exposure to estrogens is related to elevated breast cancer risk in women [1]. Both estrogen receptor ER-dependent and ER-independent mechanisms have been proposed to play an important role in carcinogenesis associated with estrogens [1]. The data that E₂ (17 β -estradiol) exerts oxidative stress resulting in various types of DNA damage has proved the direct role of estrogens in cancer initiation [1–3]. On the other hand, both experimental and clinical data support the hypothesis that oxidative stress affects ER expression and its molecular consequences [4].

Estrogen receptor α expression was documented to be essential for normal mammary gland development and was observed to be increased during breast tumorigenesis [5]. Also, ER α has long been the primary target for endocrine therapies in breast cancer. On the other hand, the significance of ER β expression in normal breast development, carcinogenesis, further progression and treatment remains unclear [5]. The clinical studies suggest that higher expression of ER β in the presence of ER α in human breast cancer is associated with a better prognosis and the probability of positive responsiveness to estrogen therapy [6]. ER β isoforms might be involved in the estrogen signaling alteration during breast tumor development. Moreover, the isoforms of ER β expressed and their cellular location might influence survival in breast cancer, but the data are inconsistent [7].

Oxidants, i.e. reactive oxygen (ROS) and nitrogen species (RNS), are known to cause a wide range of cellular effects depending on the dose and cellular environment; for example they can act as second messengers to regulate a variety of physiological processes. Oxidative damage to both nDNA and mtDNA may lead to mutations that activate oncogenes or inactivate tumor suppressor genes. ROS/RNS can cause oxidative damage to macromolecules, thus contributing to pathogenesis of age-related and chronic disorders including cancer. ROS react with polyunsaturated fatty acids, leading to formation of lipid peroxidation products, in turn leading to tumor promotion [8, 9].

Malignant cells are characterized by persistent oxidative stress. ROS/RNS can activate growth-promoting transcription factors and modulate gene expression important in proliferation and apoptosis. Oxidants may damage protease inhibitors and thus promote tumor invasion. The chronic inflammation associated with the malignant process is among others an abundant source of ROS/RNS resulting in further genetic instability [2, 3, 10].

Aerobic organisms have created a variety of antioxidant mechanisms to counterbalance the oxidative damage and thus to maintain their genomic stability. Antioxidant defense systems consist of multiple interdependent components. Intracellular system: glutathione GSH and enzymes such as superoxide dismutase (CuZn-SOD, Mn-SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRx) and glutathione-S-transferase (GST). Extracellular system: mostly proteins and low-molecular weight substances such as vitamin C, E, A, β -carotene, retinol, uric acid and bilirubin. As was suggested, in a living organism a fine balance among many antioxidants appears to be more important for the overall protective capacity of the defense machinery than the activity/concentration of a single constituent [8].

Oxidative stress, i.e. loss of the fine balance between pro- and antioxidant mechanisms favoring ROS/RNS pro-

duction, is considered to be involved in breast cancer pathogenesis [11].

Plasma total antioxidant status (TAS) estimates peroxyl-scavenging capacity of the extracellular antioxidant system, comprised of protein thiol groups (52.9%), uric acid (33.1%), vitamin C (4.7%), bilirubin (2.4%), vitamin E (1.7%) and unidentified antioxidants (5.2%) [12, 13]. Total antioxidant status was found to be under strong genetic control. Additive effects account for 50% of the phenotypic variance in TAS levels in nonsmokers and for 83% in smokers [13].

Hence, the aim of this pilot study was to evaluate the plasma total antioxidant capacity in breast cancer patients in relation to ER β expression.

Material and methods

The study group consisted of newly diagnosed consecutive breast cancer patients ($n = 41$) and controls ($n = 28$) randomly selected from benign breast disease patients admitted to the 1st Department of Surgical Oncology and General Surgery, Greater Poland Cancer Center in Poznan, Poland. On the basis of complete clinical examination, those with the following conditions were excluded from the study: diabetes mellitus, prediabetes, advanced atherosclerosis of any location, chronic liver or renal disease, any inflammatory process, malabsorption or malnutrition syndrome, alcohol abuse and malignancy other than breast cancer. The menopausal status was established on the basis of the data of last menstruation or the gynecological surgery. The studied women did not admit to use of any micronutrient supplementation. Smokers were asked not to smoke overnight before the blood collection.

Blood samples were collected from women with breast cancer and benign breast conditions after overnight fasting and stored at -80°C until assayed.

Lymph node status was established as negative (N0: without any lymph nodes involved; MO: without any distant metastases) or positive (N+: with lymph nodes involved; MO: without any distant metastases).

The clinicopathological characteristics of the studied groups of patients are shown in Table 1.

All patients were informed of the study purpose and gave written consent. The reported study was approved by the Ethics Committee of Poznan University of Medical Sciences, Poznan, Poland.

The plasma total antioxidant status was determined with the ABTS reagent (2,2'-azino-di-[3-ethylbenzthiazoline sulfate]) by colorimetric test (RANDOX Laboratories Ltd, Crumlin, United Kingdom) on StatFaxTM 1904 Plus (Awareness Technology, Inc, Palm City, FL, USA).

Estrogen receptor α , ER β , PgR and HER-2 were measured routinely in breast cancer tissue in Greater Poland Cancer Center, Poznań, Poland. Immunostaining for ER α , ER β and PgR was performed using monoclonal antibodies against ER α , PgR (DAKO Glostrup, Denmark) and polyclonal antibodies against ER β (CHEMICON International, Temecula, CA). The EnVision detection system was applied. HER-2 oncogene expression was determined with the immunohistochemical method (HerceptTestTM, DAKO, Glostrup,

Table 1. Clinical characteristics of the patient groups: with breast cancer and benign breast disease (control group)

Study group/subgroup	Number of patients
Breast cancer group	41
Menopausal status	
pre-	6
post-	35
HRT	12
Histology type	
ductal	37
lobular	3
other	1
Clinical stage:	
a) Dcis	4
T1	25
T2	11
T4	1
b) N0 (lymph node negative)	29
N+ (lymph node positive)	12
Hormonal receptors status	
ER α +, ER β +, PgR+, HER-2-	12
ER α +, ER β +, PgR+, HER-2+	7
ER α +, ER β -, PgR+, HER-2-	12
ER α +, ER β -, PgR+, HER-2+	4
ER α +, ER β +, PgR-, HER-2-	1
ER α +, ER β -, PgR-, HER-2+	2
ER α -, ER β +, PgR-, HER-2+	1
ER α -, ER β -, PgR-, HER-2-	1
ER α -, ER β -, PgR-, HER-2+	1
Smoking history (positive)	9
Control group	28
Menopausal status	
pre-	6
post-	22
HRT	10
Histology type	
fibroadenoma	10
mastopatia fibrosa and cystica	16
adenosis sclerosans	2
hyperplasia ductalis	5
Smoking history (positive)	5

HRT – hormonal replacement therapy

Denmark). HER-2 protein was considered over-expressed (positive) if the staining was classified as 3+, whereas all 2+ cases were verified by amplification of the c-erbB2 gene testing with fluorescent in situ hybridization (FISH).

Statistical analysis

Statistical analysis was performed using Statistica 10.0 for Windows (StatSoft). The results are expressed as medians (25–75% range). The distribution of values was verified by Shapiro-Wilk test. The comparisons between studied groups and subgroups were performed using non-parametric Mann-Whitney test. Spearman rank order correlations between TAS and age were calculated. The statistical significance was accepted at the level $p < 0.05$.

Results

The study demonstrated a statistically significant decrease in the plasma TAS concentration in the breast cancer patient group when compared to the controls (Fig. 1). The TAS concentration was found not to differ significantly between breast cancer subgroups in regard to the status of lymph nodes (NO vs. N+). The TAS level was not significantly different between breast cancer subgroups, either in relation only to ERβ expression (ERβ+ vs. ERβ-) or considering the steroid receptor status (ERα+, ERβ+, Pg+ vs. ERα+, ERβ-, Pg+), even in the selected lymph node negative subgroup. Similarly, HER-2 expression did not significantly affect the TAS concentration (Table 2).

The difference in the TAS level between breast cancer (lower) and control (higher) patients was maintained when the subjects were categorized in relation to the hormonal status [i.e. premenopausal, postmenopausal with and without hormonal replacement therapy (HRT)]. The TAS concentration was found to be significantly lower in the premenopausal breast cancer subgroup than in the postmenopausal group without HRT. Also, a tendency of TAS level to increase with age in both studied groups, with and without breast cancer, was observed. A positive correlation between TAS and age was found in the whole breast cancer group ($R = 0.44$, $p = 0.004$) whereas in the control group and in studied subgroups no significant correlation was observed. The age of given subgroups was similar between the cancer patients and the controls, while it was significantly different among subgroups within one study group, i.e. either with cancer or controls (Table 3).

Discussion

Oxidative stress and gene-environment interactions are considered to play a significant role in the development of breast cancer [11]. Oxidative stress can either enhance or decrease the activity/concentration of antioxidants depending on adaptive abilities of the organism and the time of exposure (acute or chronic) [14].

Decreased plasma total antioxidant capacity across patient subgroups of different age in comparison to the controls, as the present study has demonstrated, suggests consumption of plasma antioxidants by enhanced ROS/RNS production in women newly diagnosed with breast

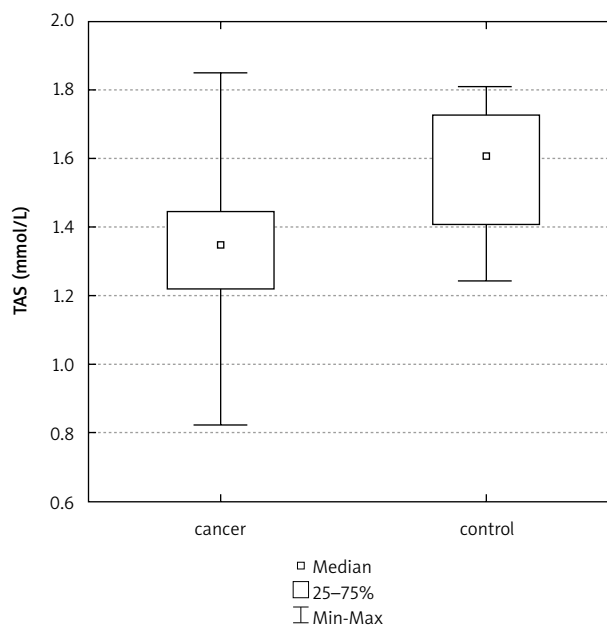


Fig. 1. The comparison of plasma total antioxidant status (TAS) between the breast cancer and the control groups

cancer. Thus, the finding supports the hypothesis of oxidative stress involvement in breast carcinogenesis.

However, the study done by Singh *et al.* revealed that plasma ferric reducing capacity (a method different than TAS) did not differ significantly between breast cancer patients before any treatment and healthy controls [15].

The results of other investigations have demonstrated altered redox status in breast cancer tissue when compared to adjacent normal tissues and to fibroadenoma. Most of the data consistently have reported increased levels of lipid peroxidation markers. Activities of cellular antioxidant enzymes, e.g. MnSOD, CuZnSOD, GPx, and the concentration of GSH, were also found to be elevated, whereas activity of CAT was determined to be either increased or decreased [16, 17].

Table 2. The plasma total antioxidant status (TAS) in the breast cancer and the benign breast disease (control) groups in relation to the lymph node status, ERβ status, steroid receptor status and HER-2/neu expression. The results are expressed as medians (25–75% range)

Study group/subgroup	TAS (mmol/l)
Breast cancer	1.35 (1.22–1.44)*
status of lymph node: negative (NO)	1.33 (1.22–1.42)
status of lymph node: positive (N+)	1.35 (1.27–1.55)
ERβ positive (+) (n = 21)	1.33 (1.27–1.43)
ERβ negative (-) (n = 20)	1.36 (1.20–1.48)
ERα+, ERβ+, PgR+ (n = 19)	1.30 (1.24–1.43)
ERα+, ERβ-, PgR+ (n = 16)	1.36 (1.20–1.48)
NO: ERα+, ERβ+, PgR+ (n = 13)	1.29 (1.27–1.43)
NO: ERα+, ERβ-, PgR+ (n = 13)	1.35 (1.18–1.38)
HER-2 negative (-) (n = 26)	1.34 (1.22–1.38)
HER-2 positive (+) (n = 15)	1.36 (1.24–1.59)
ERα+, ERβ+, PgR+, HER-2- (n = 12)	1.31 (1.27–1.36)
ERα+, ERβ-, PgR+, HER-2- (n = 12)	1.36 (1.16–1.56)
Controls	1.61 (1.41–1.73)*

* $p = 0.0001$

Table 3. The plasma total antioxidant status (TAS) and age in the breast cancer and the control groups in relation to the hormonal status. The results are expressed as medians (25–75% range)

Parameter	Breast cancer	Controls	p-value
TAS (mmol/l)	1.26 (1.06–1.26)*	1.41 (1.38–1.56)	0.01
Age (years)	42.0 (41.0–45.0) ^{1,2}	37.5 (36.0–42.0) ^{4,5}	NS
TAS (mmol/l)	1.30 (1.20–1.29)	1.60 (1.42–1.69)	0.04
Age (years)	52.0 (48.0–58.5) ^{1,3}	57.0 (54.0–58.0) ⁴	NS
TAS (mmol/l)	1.42 (1.24–1.67)*	1.71 (1.57–1.75)	0.01
Age (years)	62.0 (58.0–70.0) ^{2,3}	58.0 (55.5–68.0) ⁵	NS

^{1,2,3,4,5} $p < 0.05$

HRT – hormonal replacement therapy; TAS – total antioxidant status

Similarly, studies on blood oxidant-antioxidant status in breast cancer patients revealed higher levels of superoxide radical and lipid peroxidation products [18]. In breast cancer patients CuZnSOD, CAT, GPx and GST activity along with GSH concentration was reported to be increased in comparison to fibroadenoma patients [19]. In different studies, CuZnSOD, CAT, GPx, GST, GSH and vitamin C and E were found to be decreased when compared to both fibroadenoma and healthy controls [20, 21]. It was concluded that even upregulated cellular antioxidant enzyme activities do not protect macromolecules (e.g. lipids) from the consequences of oxidative stress in the course of breast malignancy. The above findings also might confirm enhanced consumption of plasma antioxidant micronutrients. Moreover, higher levels of plasma total antioxidant capacity determined with a different method (Trolox-equivalent antioxidant assay) and plasma micronutrients/metabolite with strong antioxidant properties (e.g. β -carotene, retinol and bilirubin) were previously reported to be associated with reduced risk of breast cancer [22]. To what extent oxidative stress detected in breast cancer patients is a causative agent and to what extent it is the result of the malignant process remains to be elucidated in the context of the possible preventive intervention.

Estrogen receptor β was reported to play an important role in cellular protection against oxidative stress. Tamoxifen-liganded ER β was observed to activate transcriptionally the major detoxifying enzyme NAD(P)H:quinone oxidoreductase and to protect against E₂-induced oxidative DNA damage in breast epithelial cell lines [23]. The present investigation failed to demonstrate any significant difference in the plasma TAS concentration between breast cancer subgroups in relation to the ER β expression considered either alone or in conjunction with other steroid receptors or along with HER-2/neu expression. However, a striking tendency was observed towards higher TAS concentration in all subgroups with ER β negative expression also in the same receptor status and lymph node negative patients.

Oxidative stress can change the structure and function of ER and PgR, influencing the biology and clinical outcome of ER-positive breast malignancy [2, 24]. The data suggest that ROS/RNS production may regulate both ER α and β expression and therefore can influence a variety

of key molecular mechanisms. ROS at low physiological concentrations significantly increased ER β levels but had no effect on ER α in human breast cancer cells [24]. In our study, polyclonal antibodies against ER β were used. The determination of ER β isoforms along with other parameters of redox status (i.e. intracellular antioxidant enzymes and markers of oxidative damage to macromolecules) might enable better understanding of their mutual influence. The influence of redox status on ER function seems to be worth further exploring in the context of its possible role in anti-estrogen breast cancer prevention and treatment outcomes.

Oxidative stress is considered to play a very important role in the aging process. In the present work we found higher TAS concentration in postmenopausal women without HRT than in premenopausal breast cancer patients, whereas the difference among respective subgroups of healthy controls was not significant. The observed changes in TAS may be associated with the increasing age of patient subgroups and thus increased ROS/RNS production. That explanation might be supported by our finding of a positive correlation between TAS level and age in the cancer group. Wang *et al.* demonstrated that TAS levels remain relatively constant across age groups in women with a slight tendency to be higher in the 60-year-old than in the 40-year-old group [13]. The observed changes in TAS might also be related to different estrogen status in patient subgroups. In our opinion, the correct interpretation of the differences in TAS related to age and estrogen status in both breast cancer and healthy women could be done only along with levels of markers of oxidative damage to macromolecules. Otherwise, we can speculate only whether increased TAS level results from increased production of ROS/RNS inducing increased activity of plasma antioxidants or from decreased production of ROS/RNS, hence less consumed extracellular antioxidants.

In summary, using a simple and relatively cheap method for the plasma TAS, no significant difference between breast cancer subgroups in relation to ER β expression was found. An observed tendency towards higher TAS level in all ER β negative breast cancer patients seems to be worth further exploring, especially along with markers of oxidative damage to macromolecules for the complete estimation of redox imbalance and on a bigger group of breast

cancer patients to determine whether it could help in personalization of primary and/or secondary prevention of breast cancer.

The authors declare no conflict of interests.

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