Carbonyl and oxidative stress in patients with breast cancer – is there a relation to the stage of the disease?

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Oxidative and carbonyl stress may, on one hand, contribute to the progression of cancer, on the other hand, they may have some antiproliferative effects.

We examined serum levels of AGEs (advanced glycation end-products), CML (carboxymethyllysine) and AOPP (advanced oxidation protein products) in 86 patients with breast cancer subdivided based on the clinical stage (TNM classification), histologic grading, expression of hormonal and C-erb B2 receptors and in 14 healthy age-matched women as controls.

Breast cancer patients had higher serum concentrations of AGEs (325,581 ± 66,037 vs. 271,322 ± 34 826 AU, $p < 0,01$) even in the early stage of the disease; patients with advanced breast cancer (stage III and IV) had significantly higher both AGEs and AOPP (113.0 ± 44.9 vs. 78.1 ± 28.4 μmol/l, $p < 0.05$) levels, not only compared to controls, but also compared to stages I and II. Serum levels of AOPP were higher in patients having only weakly positive expression of C-erb 2/Her-neu compared to controls and the patients having the highest C-erb2/Her-neu expression. Serum concentrations of AGEs in patients with breast cancer correlated with the age and also with the serum concentration of AOPP.

In conclusion: breast cancer patients had an early increase of AGEs (marker of the carbonyl stress) followed by further increase of AGEs and elevation of AOPP (marker of oxidative stress) in patients with progressive disease.

As the clinical significance of these observations is currently uncertain further studies are clearly warranted, especially with respect to their potential therapeutic implications.

Key words: advanced glycation end-products, advanced oxidation protein products, breast cancer, carbonyl stress, carboxymethyllysine, oxidative stress

Oxidative stress characterized by the imbalance between the concentration of the reactive oxygen (e.g. superoxide, hydrogen peroxide, hydroxyl radical) and nitrogen (e.g. peroxynitrite) species and the activity of antioxidant enzymes (e.g. superoxiddismutase, glutathioperoxidase) and antioxidant substrates (e.g. vitamin A, C and E) results in the damage to the lipids, carbohydrates and/or nucleic acids and plays an important role in the pathogenesis of some chronic diseases and their complications (e.g. diabetes, atherosclerosis, chronic renal failure, rheumatoid arthritis, or Alzheimer's disease [1, 2]).

Damage to the proteins by the oxidative stress results in the formation of the advanced oxidation protein products (AOPP [3]). Structure of the AOPP is not well defined, but may be formed mostly (in relation to its molecular weight) by the oxidatively modified albumin and its aggregates. AOPP tightly correlate with dityrosine as a marker of the protein oxidation, pentosidine (a species of AGEs formed also with the participation of the oxidative stress), neopterin (a marker of the monocyte activation), IL-1ra, TNF-α and soluble TNF receptor (markers of inflammation), but, similarly as AGEs, AOPP do not correlate with the product of lipoperoxidation malondialdehyde and are not probably related to the lipoperoxidation.

Oxidative stress is closely related to the carbonyl stress which is characterized by the increase of the reactive carbonyl compounds due to their increased formation and/or due to their decreased degradation and elimination [4]. Reactive carbonyl compounds may contribute to the formation of the

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advanced glycation end-products (AGEs) and the advanced lipoperoxidation end-products (ALEs).

Advanced glycation end-products (AGEs) are formed in the organism under normal condition and their formation increases with the age. Formation of AGEs is pathologically increased due to hyperglycaemia in diabetes and AGEs also accumulate in uremia. AGEs is a heterogeneous group of compounds which can be assessed using their characteristic fluorescent spectrum (fluorescent AGEs) or by ELISA using polyclonal antibodies [5]. Pentosidin, carboxymethyllysine (CML), pyrraline, imidazolone, glyoxallysine dimer – GOLD) and metydioxallysine dimer (MOLD) belongs among well characterized AGEs. These substances may be evaluated using ELISA with specific monoclonal antibodies or using high performance liquid chromatography or gas chromatography coupled to mass spectrometry.

Tissue accumulation of AGEs results in different toxic effects and contributes to the development of diabetic microangiopathic complications (nephropathy, neuropathy, and retinopathy). AGE-induced damage to the endothelium with the modification of LDL contributes to the acceleration of atherosclerosis.

AGEs may exert their effect also by the activation of its specific receptor – RAGE (receptor for advanced glycation end-products). Binding of AGEs to RAGE stimulates oxidative stress, activates nuclear factor NF-kappa B and stimulates the formation of cytokines, growth factors and the expression of adhesion molecules with subsequent stimulation of cellular proliferation, increase of vascular permeability and induction of the migration of macrophages [6, 7, 8].

Oxidative and carbonyl stress may contribute to the process of cancerogenesis [9, 10]. E.g. hydroxynonenal, the product of lipoperoxidation, may link the receptors for the epidermal growth factor (EGFR) and stimulate apoptosis. One of the reactive carbonyl compounds, methylglyoxal, may increase the synthesis of VEGF (vascular endothelial growth factor) and induce apoptosis of leukemic T-cells, glyoxal may also activate some proteinkinases, e.g. c-src and increase the intracellular phosphorylation of tyrosine residues.

Tumors are characterized by the increased uptake of glucose and activated glycolysis with putatively increased non-enzymatic glycation of proteins. Presence of AGEs, specifically CML and argpyrimidine has been demonstrated in the tissue of the several human tumors by specific antibodies [11].

The aim of our study was to find out if the markers of carbonyl (AGEs, CML) and oxidative (AOPP) stress are increased in patients with breast cancer compared to healthy controls and if their serum levels relate to the biologic behaviour of the tumor.

Patients and methods

Patients. We examined 86 patients with breast cancer regularly followed in the Department of Oncology of the General Faculty Hospital in Prague. All examined patients were after the breast surgery and after the end of the adjuvant chemotherapy (at minimum half year after the diagnosis of breast cancer), 61 of them (patients presenting with positive estrogen receptors) were at the time of examination under hormonal treatment (tamoxifen in 49 patients, aromatase inhibitor in 12 patients).

Patients were subdivided based on the clinical stage (TNM classification – the size of tumour, involvement of regional lymphatic nodes and the presence of distant metastases –localized – stage I, locally progressive – stage II, advanced locally progressive – stage III, and generalized – stage IV), dedifferentiation of the tumor (histologic grading), expression of hormonal receptors (positivity means at least 5% of positive tumor cells for estrogen or progesteron receptors immunohistochemically) and the expression of the C-erb B2/Her2-neu – receptor for the epidermal growth factor, increased expression of this receptor in the tumor is a negative prognostic sign and the predictive factor for the treatment with the trastuzumab – positivity was assessed semiquantitatively immunohistochemically using Hercept test by Dako). None of examined patients suffered from diabetes mellitus and serum creatinine did not exceed 120 μmol/l in any of them.

As controls we examined 14 healthy age-matched women.

Study was performed in keeping with the principles of the Declaration of Helsinki and was approved by the Ethical Committe of the General Faculty Hospital.

All participating subjects signed the informed consent with the sampling of their blood and the examination of the evaluated parameters.

Blood used for the examination was taken into the tubes without the addition of anticoagulants, centrifuged for 10 minutes with 1450 g and serum was frozen to -80 degree Celsius. Analysis of all samples was performed within 6 months.

Laboratory parameters. Measurement of AGEs (advanced glycation endproducts) AGEs were measured spectrofluorimetrically (excitation 350 nm, emission 435 nm) in the serum diluted by the phosphate buffer according to Henle [12] and Munch [5] (spectrofluorimetre Fluoromax-3, Jobin Yvon Horiba, USA) and are expressed in the arbitrary units (AU).

Measurement of carboxymethyllysine. Carboxymethyllysine was measured immunochemically. After the digestion of the serum samples by the proteinase K and the inactivation of the protease in 80 degrees of Centigrade, AGE-CML was assessed using the method of the competitive ELISA with the specific anti-CML monoclonal antibody 4G9. 6-(N-carboxymethylamino)capronic acid (Roche Diagnostics GmbH, Penzler, Germany) was used for the calibration. Concentration of carboxymethyllysine is expressed in μg/l.

Measurement of AOPP. Advanced oxidation protein products were assessed according to Witko-Sarsat [3]. 200 μl of serum diluted 1:5 by the phosphate buffer, pH 7.4, or by 200 μl of chloramine T (0 – 100 μmol/l) for calibration, or by 200 μl of the phosphate buffer as a blank was applied into the single holes of the microtitration plate. 10 μl of the 1.16 mol/l potas-

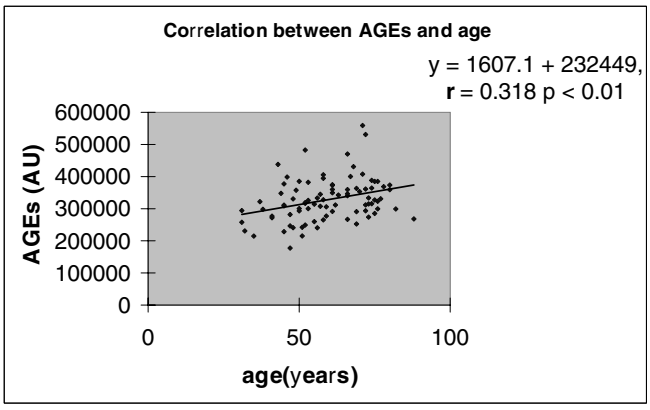

Figure 1. Correlation between AGEs and age in patientw with breast cancer

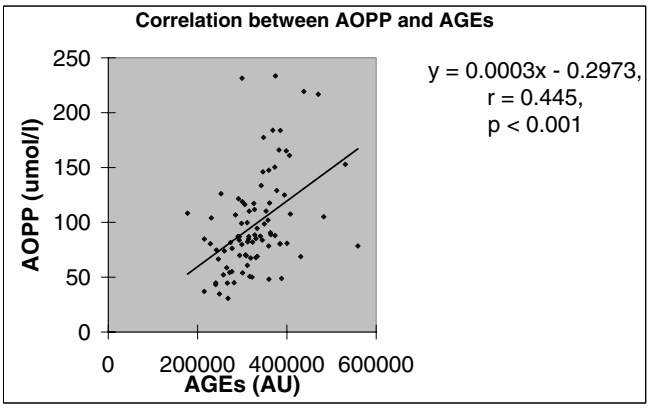

Figure 2. Correlation between AOPP and AGEs in patients with breast cancer

sium iodide and 20 µl of acetic acid were then added and the absorbance in 340 nm was measured immediately (photometre Multiscan Ascent, Labsystems, Finland). Concentraion of AOPP was related to the calibrator and expressed in mmol/l.

Statistical analysis. Results of the examination are expressed as a mean ± standard deviation. The groups were compared using the analysis of variance (ANOVA) and Kruskal-Wallis test. Relation between the parameters was found out using the Spearman correlation coefficient.

Difference was supposed to be significant if $p < 0.05$ [13].

Results

Clinical characteristic of the patients with the breast cancer. Median age of the patients with the breast cancer and the helathy women was comparable (58.7 ± 13.0 vs. 57.1 ± 4.1 years – Table 1).

Patients were subdivided into subgroups based on the clinical stage, grade, presence of estrogen receptors and the expression of C erb B2 (Her2-neu) receptors.

Out of 86 patients 24 presented with the clinical stage 1, 44 with the clinical stage 2, 13 in clinical stage 3 and only 5 clinical stage 4. 13 patients were assessed as grade 1, 51 as grade 2 and 18 as grade 3 (Tables 2 and 3).

Estrogen receptors evaluated in the histologic samples of the breast cancer using immunohistochemistry were positive in 61 pts and negative in 23 pts. C-erb B2 (Her-neu) expression (evaluated immunohistochemically – Hercept-test, Dako) was negative (Erb 0) in 24 pts, the positivity in the remaining 62 pts was assessed semiquantitatively as Erb 1 in 17 pts, Erb 2 in 25 pts and Erb 3 in 20 pts.

Concentration of AGEs, CML and AOPP in patients with breast cancer in relation to the clinical classification and the expression of estrogen and C erb 2/Her-neu receptors.

Patients with breast cancer had higher serum concentrations of AGEs; serum concentrations of CML and AOPP, however, did not differ in patients with breast cancer from control age-matched subjects (table 1).

Patients with advanced breast cancer (clinical stage 3 and 4) had significantly higher AGEs and AOPP not only compared to controls, but also compared to the patients in the clinical stages 1 and 2 (table 2).

There was no significant difference in the serum concentrations of AGEs, CML and AOPP in the different subgroups of patients with breast cancer based on the grade of the disease and positive or negative expression estrogen receptors.

Patients with only a weakly positive expression of C-erb 2/Her-neu (erb 1) had, compared to controls, higher serum concentrations of AOPP, in the opposite the patients with the highest expression of C-erb 2/Her-neu (erb 3) had, compared to patients with only weakly positive expression of C-erb 2/ Her-neu significantly lower concentrations of AOPP.

Serum concentration of AGEs correlated in patients with breast cancer with the age ($r = 0.318$ $p < 0.01$, fig. 1) and also with the serum concentration of AOPP ($r = 0.445$, $p < 0.001$, fig. 2).

Discussion

Patients with breast cancer after local surgical treatment and adjuvant chemotherapy had even in the early stage I (disease localized to the breast, with tumor smaller than 2 cm) increased plasma levels of AGEs. Patients in clinical stages

Table 1 Median age and parameters of carbonyl and oxidative stress in patients with breast cancer

	controls	breast cancer
Age (years)	57.1 ± 4.1	58.7 ± 13.0
AGEs (AU)	271 322 ± 34 826	325 581 ± 66 037 **
CML (µg/l)	547.3 ± 95.0	563.1 ± 178.5
AOPP (µmol/l)	78.1 ± 28.4	94.9 ± 40.9

Table 2 Parameters of carbonyl and oxidative stress in different stages of breast cancer

	Number	**AGEs** (AU)	**CML** (μg/l)	**AOPP** (μmol/l)
Controls	14	271 322 ± 34 826	547.3 ± 95.0	78.1 ± 28.4
breast cancer	86	325 581 ± 66 037 **	563.1 ± 178.5	94.9 ± 40.9
stage I	24	331 734 ± 62 072 **	576.6 ± 177.6	89.3 ± 40.7
stage II	44	306 813 ± 57 405 *	574.3 ± 156.8	92.3 ± 41.4
stage III	13	**370 174 ± 82 870 *** ##**	513.5 ± 216.0	**110.6 ± 37.4 ***
stage IV	5	**368 135 ± 46 635 *** #**	528 ± 308.4	**119.1 ± 50.6 ***
stage I-II	68	306 813 ± 57 405 *	575.1 ± 163.8	91.2 ± 41.2
stage III-IV	18	**369 607 ± 66969 *** ++**	517.5 ± 281.9	**113.0 ± 44.9 *+**

* p < 0.05, ** p < 0.01, *** p < 0.001 compared to controls,
# p < 0.05, ## p < 0.01, compared to stage II
+ p < 0.05, ++ p < 0.01, compared to stage I-II

Table 3 Parameters of carbonyl and oxidative stress in patients with breast cancer according to the grade and expression of estrogen and Her/Neu receptors

	Number	**AGEs** (AU)	**CML** (μg/l)	**AOPP** (μmol/l)
Controls	14	271 322 ± 34 826	547.3 ± 95.0	78.1 ± 28.4
breast cancer	86	325 581 ± 66 037 **	563.1 ± 178.5	94.9 ± 40.9
grade 1	13	319 552 ± 60 156 *	528.8 ± 169.0	96.1 ± 35.1
grade 2	51	329 853 ± 69 864 **	556.7 ± 175.7	94.5 ± 43.9
grade 3	18	328 858 ± 63 422 **	594 ± 200.3	102 ± 38.4
ER1	61	328 419 ± 70 761 **	550.3 ± 185.5	97.7 ± 41.7
ER2	23	321 992 ± 53 545 **	596.2 ± 166.3	89.2 ± 40.2
Erb 0	24	334 261 ± 79 332 **	609 ± 190.0	96.7 ± 38.5
Erb 1	17	331 804 ± 54 052 **	501.8 ± 170.2	**115.4 ± 56.4 ***
Erb 2	25	312 635 ± 55 290 *	530.0 ± 180.0	94.4 ± 35.1
Erb 3	20	331 045 ± 68 098 **	594.5 ± 156.6	**76.9 ± 28.6 &**

* p < 0.05, ** p < 0.01, *** p < 0.001 compared to controls,
& p < 0.05 compared to Erb 1

III-IV had serum levels of AGEs higher than patients in clinical stages I-II. We were not able to demonstrate any relation of the serum levels of AGEs either to the grade of the tumor, or to the expression of both the estrogen and C erb/Her/Neu receptors.

The presence of AGEs in the tissue of several human tumors including breast cancer has been demonstrated using specific antibodies [11]. AGEs may stimulate the proliferation of tumor cells by the activation of the receptor for advanced glycation end-products (RAGE [8]).

Binding of AGEs or other ligands to RAGE results in the activation of the key mediators of the proliferation and inflammation, e.g. p21ras, MAP kinases, NF-kappaB and cdc42/rac. One of the most important ligands of RAGE is the polypeptide amphoterin. Blockade of the amphoterin/RAGE system (by the suppression of p44/p42, p38 and SAP/JNK MAP proteinkinases) lowers in mice the migration and invasivity of tumor cells and possibly also cell proliferation and the production of tissue metalloproteinases [14].

In patients with colorectal cancer the expression of RAGE increases with the progression of the tumor and the patients with the concomitant expression of amphoterin and RAGE had worse outcome than patients who expressed only amphoterin [15]. Growth, migration and invasivity of the cells of the colorectal cancer may be blocked in the cells expressing both RAGE and amphoterin by the amphoterin anti-sense oligonucleotides [16] and also by AGE-modified albumin. AGE-modified albumin may impair the phosphorylation of some proteinkinases (extracellular signal-regulated kinase-1/2, Rac1 a AKT) and the formation of metalloproteinase 9. On the other hand, compared to amphoterin, AGE-modified albumin may stimulate more NO synthase and NF kappa B.

Increased expression of RAGE was demonstrated also in most cell lines of the gastric cancer, correlating with the dedifferentiation of the adenocarcinoma, depth of tumor invasion and the presence of metastasis in lymph nodes [17]. Expression of RAGE has also correlated with the invasivity of the cell lines derived from human gallbladder cancer [18]. In hormonally independent cell lines derived from the prostate cancer growth and invasivity of the cancer cells could have been stimulated by the administration of AGE-modified albumin [19]. Expression of RAGE also correlated with the metastatic potential of the cells derived from human pancreatic cancer [20]. On the other hand, in the cells of human

non-small cell lung cancer, expression of RAGE was decreased, probably as a consequence of the high concentrations of amphoterin amphoterin [21]. Induced expression of RAGE decreased the rate of growth of the tumor cells [22] and limited the proliferation of the lung fibroblasts [23].

Expression of RAGE was demonstrated also in the tissue of breast and lung cancer using the microarrays [24].

RAGE could thus become the promising target of the tumor cell directed antitumor treatment. Currently it is unclear which one of the two putative effects of increased serum levels of AGEs may prevail in patients with cancer (including breast cancer): RAGE-mediated stimulation of the growth and invasivity of the tumour, or the competitive inhibition of the stimulatory effect of amphoterin, or the decreased expression of RAGE by the tumour cells.

One of the specific AGEs formed during the carbonyl stress is carboxymethyllysine (CML). CML binds to nuclear proteins (e.g. histons). Histons with bound CML are only slowly degraded in the proteasomes. The activity and concentration of proteasomes are increased by CML-modified histones. Accumulated CML-modified histones may influence the gene expression and contribute to the tumor transformation of keratinocytes [25]. Binding of CML to RAGE may also stimulate tumor cells proliferation by the activation of proteinkinases (e.g. p44/42 – ERK $^1/_2$ MAP kinases – [26]).

Serum levels of carboxymethyllysine (CML) were not increased in our patients with breast cancer compared to controls. There was a non-significant trend to the decrease of serum concentrations of CML in patients in clinical stages III – IV compared to patients in clinical stages I – II.

AOPP are increased in patients with chronic renal failure, diabetes and their role in the oxidative modification of LDL and the pathogenesis of atherosclerosis has been also suggested.

Oxidative stress, namely oxidative damage to DNA may play a critical role in the cancerogenesis [9, 10, 27]. Protein oxidation correlated with morphologic hyperplastic changes in the model of estrogen-induced cancerogenesis [28].

Serum levels of AOPP were significantly increased only in our patients with advanced breast cancer in clinical stage III – IV. Serum levels of AOPP significantly correlated with the serum levels of AGEs suggesting the common mechanisms of the formation of both types of modified molecules.

We have no apparent explanation for the finding of the decreased serum concentrations of AOPP in patients with highest expression of C erb/Her neu. Oxidative stress with the glutathion depletion may lead to the accumulation of the ceramide with subsequent stimulation of apoptosis. In the same moment, oxidative stress may block the degradation of the EGF receptor and thus contribute to the tumor proliferation [29]. Supposedly, oxidative stress may have (at least from the view of the C-erb/Her neu expression) antitumor effect.

To our best knowledge we were the first to study serum levels of AGEs, CML and AOPP in patients with breast cancer. We were able to demonstrate that patients with breast cancer had since the early stage I (with no respect to the grade and the expression of both estrogen and C erb 2/Her-neu receptors) increased serum concentrations of AGEs. Serum levels of AGEs were further increased in patients with clinical stages III-IV compared to the patients with clinical stages I-II.

We were not able to demonstrate, however, any increase of CML in patients with breast cancer (with no relation to the biologic activity of the disease) with only a non-significant trend to lower concentrations of CML in patients in clinical stages III-IV compared to the clinical stages I-II.

AOPP was increased only in patients in the clinical stages III-IV. AOPP was highest is patients with Erb 1 and lowest in patients with Erb 3.

The early increase of AGEs (marker of the carbonyl stress) in patients with breast cancer was followed by the further increase of AGEs and elevation of AOPP (marker of oxidative stress) in patients with progressive disease.

Clinical significance of these observations is currently uncertain. On one hand, both carbonyl and oxidative stress may contribute to the progression of the disease, on the other hand, increase of AGEs and AOPP in patients with advanced breast cancer may have antiproliferative effects (competition of AGEs with amphoterin for binding with RAGE, decreased expression of RAGE due to increased concentration of AGEs observed in advanced stages of some tumors, decreased expression of cErb/Her2-neu due to the oxidative stress).

Further study of the influence of carbonyl and oxidative stress on the biologic behaviour of the breast cancer is thus warranted, especially with the respect to its potential therapeutic implications.

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