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Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients

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Keywords:	antioxidant effects, cancer susceptibility, DNA damage, DNA repair, genetic polymorphisms



For Peer Review

Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients

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Running title: Oxidative stress and 8-oxoGua repair in CRC patients

Key words: 8-oxoguanine, DNA repair, colon cancer, polyps, antioxidants

Abstract

Oxidative stress is involved in the pathogenesis of colon cancer. We wanted to elucidate at which stage of the disease this phenomenon occurs. In the examined groups of patients with colorectal carcinoma (CRC, n=89), benign adenoma (AD, n=77), and healthy volunteers (controls, n=99) we measured: vitamins A, C and E in blood plasma, 8-oxodG and 8-oxoGua in leukocytes and urine, leukocyte 8-oxoGua excision activity, mRNA levels of APE1, OGG1, MTH1 and *OGG1* polymorphism. The vitamin levels decreased gradually in AD and CRC patients. 8-OxodG increased in leukocytes and urine of CRC and AD patients. 8-OxoGua was higher only in the urine of CRC patients. 8-OxoGua excision was higher in CRC patients than in controls, in spite of higher frequency of the *OGG1* Cys326Cys genotype, encoding a glycosylase with decreased activity. mRNA levels of OGG1 and APE1 increased in CRC and AD patients, which could explain increased 8-oxoGua excision rate in CRC patients. MTH1 mRNA was also higher in CRC patients. The results suggest that oxidative stress occurs in CRC and AD individuals. This is accompanied by increased transcription of DNA repair genes, and increased 8-oxoGua excision rate in CRC patients, which is, however, insufficient to counteract the increased DNA damage.

Introduction

Colorectal cancer (CRC) is one of the most frequent causes of death in western countries. The most important etiological factors of sporadic colorectal tumors are inflammation, fat metabolism, tobacco smoking as well as consumption of meat and alcohol (1). Inflammation is associated with the release of large amounts of reactive oxygen species (ROS) (2), leading to oxidation of nucleic acids, proteins and lipids. Many epidemiological studies report an inverse association between vegetable and fruit consumption and occurrence of colon cancer (3). One of the possible mechanisms of the protective effect of such food constituents as vitamins A, C and E is by exerting antioxidative activities, by scavenging free radicals and preventing DNA damage. Oxidatively damaged DNA has been blamed for the physiological changes associated with degenerative diseases such as cancer (4,5). A plethora of damaged DNA bases are formed upon ROS attack on genetic material, several of them reveal strong promutagenic properties (6). One of the major and best studied is 8-oxo-7,8-dihydroguanine (8-oxoGua), a typical biomarker of oxidative stress, which may play a role in carcinogenesis (7). The presence of 8-oxo-7,8-dihydroguanosine (8-oxodG) residues in DNA leads to GC→TA transversions (8). 8-OxodG is formed in DNA either *via* direct oxidation of nucleic acids or can be incorporated from the nucleotide pool by DNA polymerases, the latter process being an important source of DNA oxidation and genome instability (9,10). Incorporation of 8-oxodGTP into DNA by DNA polymerases is limited by the activity of MTH1 phosphohydrolase, which hydrolyzes 8-oxodGTP to 8-oxodGMP (11). 8-OxodGMP is subsequently dephosphorylated by nucleotidase and removed from the cell (12). Many observations indicate a direct correlation between 8-oxodG formation and carcinogenesis *in vivo* (7,13-16).

To counteract the deleterious effect of oxidatively damaged DNA, all organisms have developed several DNA repair pathways. Excision of 8-oxoGua from DNA is accomplished

1
2
3 mainly by base excision repair. Several DNA glycosylases, which specifically recognize and
4 remove 8-oxoGua in human cells have been described, the major one apparently being OGG1
5
6 DNA glycosylase (17). Human OGG1 glycosylase recognizes and excises several lesions
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8 from oligodeoxynucleotides with single DNA damages, including 8-oxoGua, FapyGua, Me-
9
10 FapyGua, 8-oxoAde (18,19). The murine enzyme was also shown to excise 8-oxo-inosine,
11
12 O⁶-methyl-8-oxoGua and 8-amino-Gua (20). However, when γ -irradiated DNA was used as a
13
14 substrate, pure human OGG1 liberated only 8-oxoGua and FapyGua, as measured by
15
16 GC/IDMS (18). FapyGua, but not 8-oxoGua, are also eliminated from DNA by NEIL1 DNA
17
18 glycosylase (21). Both these DNA lesions show strong mutagenic activity in mammalian cells
19
20 (22). Numerous experimental data suggest a decrease in DNA repair efficiency in cancer
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22 patients (23-27). One possibility is that such a decrease may be caused by the presence of
23
24 polymorphic forms of DNA repair genes, which encode proteins with reduced enzymatic
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26 activities (26). Several polymorphic changes in the *OGG1* gene have been described, with the
27
28 most common being Ser326Cys (28). Polymorphic Cys326 OGG1 protein was found to have
29
30 a lower enzymatic activity, both when 8-oxoGua was excised from oligodeoxynucleotides
31
32 (29), and when 8-oxoGua and FapyGua were liberated from γ -irradiated DNA by pure
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34 Cys326 or Ser326 OGG1 enzyme (18,28). It was suggested that the presence of two *OGG1*
35
36 326Cys alleles may confer an increased risk of lung, prostate and nasopharyngeal cancer (30-
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38 32), but no association with the risk for colon cancer (33,34). Our and other functional studies
39
40 report a decrease in 8-oxoGua excision rate in lung (23,24) and head and neck cancer patients
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42 (25). Such a decrease in repair rate and simultaneous increase in 8-oxodG and FapydG levels
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44 in cellular DNA favors a pro-oxidant state and may accelerate the acquisition of mutations in
45
46 critical genes leading to cancer. Such an idea is basically derived from the observation that a
47
48 pro-oxidant environment is characteristic for advanced stages of cancer. However, it is not
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50 clear whether increased oxidative stress/oxidatively damaged DNA is merely the result of the
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3 disease or whether it plays a role in cancer development. This prompted us to investigate the
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5 broad range of biomarkers: oxidatively damaged DNA, the status of antioxidant vitamins, and
6
7 the repair of 8-oxoGua in colon cancer patients, in individuals developing benign
8
9 adenomatous polyps and in healthy controls. We observed an increase in oxidatively damaged
10
11 DNA and decreased antioxidant defense in leukocytes of colon cancer patients but also in
12
13 adenoma individuals, of which some may be at very early stages of CRC development.
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15 Increased oxidative stress stimulates the 8-oxoGua excision rate, although this stimulation is
16
17 insufficient to counteract oxidative damage to DNA.
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27 **Materials and methods**

28 *Materials*

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30 T4 polynucleotide kinase and [γ -³²P]ATP were from GE Healthcare. Oligodeoxynucleotide
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32 (40 nt) containing a single 8-oxodG at position 20 in the sequence 5'-d(GCT ACC TAC CTA
33
34 GCG ACC **TXC** GAC TGT CCC ACT GCT CGA A)-3', where **X** indicates 8-oxodG was
35
36 obtained from Eurogentec Herstal, (Herstal, Belgium). A complementary
37
38 oligodeoxynucleotide containing dC opposite 8-oxodG was synthesized in the
39
40 Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS (Warsaw, Poland).
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48 *Study group*

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50 This case-control study was conducted in three groups. The control group consisted of 99
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52 healthy individuals (44 male and 55 female) of median age 55 years (range 42 to 65 years).
53
54 The polyp individuals group (AD, n=77, 28 serrated adenomas and 49 adenomas) comprised
55
56 38 females and 39 males with a median age of 60 years (range 32 to 83). The colorectal
57
58 cancer patient group (CRC, n=89) comprised 45 males and 44 females (median age 62 years,
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1
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3 range 27 to 90). All participants were Caucasians and there were no relatives among them. All
4
5 individuals participating in the study were recruited through the hospital (Collegium
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7 Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland) and were examined by
8
9 colonoscopy. The control group was recruited from individuals undergoing routine colon
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11 cancer screening, in whom colonoscopy revealed no cancer or polyps. All the subjects, when
12
13 recruited to the study, filled in a questionnaire concerning demographic data, smoking, diet
14
15 and medical history. Interviewees were asked to estimate the average frequency of
16
17 consumption of various dietary items in the year preceding the interview. The majority of
18
19 them reportedly consumed 3 servings of fruit and vegetables and about 250 g of meat and fat
20
21 per day. To make the group even more homogenous, the subjects who reported the extreme
22
23 consumption, as well as those who reported supplementation within the last month were
24
25 excluded from the study. The questionnaire was administered by the team physician (Dr.
26
27 Banaszkiwicz). The control group was chosen to maximally match the patient groups and
28
29 adenoma individuals by age, sex, diet (consumption of fat, carbohydrates and vitamin intake),
30
31 body weight, and smoking status. Among healthy volunteers, AD individuals and CRC
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33 patients, two groups were distinguished in relation to their smoking status, namely those who
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35 had never smoked and smokers who consumed 20 or more cigarettes per day.
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44 Blood and urine was drawn from CRC and AD patients at diagnosis, and from control
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46 individuals, when they were recruited to the study following colonoscopy verifying they were
47
48 free of any morphological changes within the colon and rectum. Diagnoses of all polyps and
49
50 adenocarcinomas were confirmed by histological examination. All the CRC patients had
51
52 histologically proven adenocarcinomas at: A (n=4), B1 (n=25), B2 (n=13), C1 (n=3), C2
53
54 (n=27) and D (n=17) stage according to Duke's scale with Astler-Coller modification. In 57
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56 cases, cancer developed in the colon, and in 20 patients, in the rectum. In the majority of
57
58 cases only one tumor was identified. The information concerning tumor histology and patient
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3 questionnaire responses were blinded to all investigators (with the exception of the team
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5
6 physician) until after the statistical analysis was completed.
7

8
9 The patients were not treated with any anticancer drugs or vitamins during the time
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11 from the diagnoses until surgery (up to 4 weeks).
12

13
14 The study was conducted in accordance with the Helsinki Declaration and was
15
16 approved by the medical ethics committee of Collegium Medicum, Nicolaus Copernicus
17
18 University, Bydgoszcz, Poland. All participants signed informed consent.
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21 22 23 *Determination of plasma vitamins A, E, C and uric acid concentration by HPLC*

24
25 Quantification of vitamin E (α -tocopherol), vitamin A (retinol), vitamin C (ascorbic acid) and
26
27 uric acid by HPLC technique was as previously described (35). Briefly, for vitamin A and E
28
29 measurement, freshly prepared or freshly thawed plasma samples were mixed with 200 μ l of
30
31 HPLC-grade water and 400 μ l of ethanol in order to precipitate proteins. For vitamin
32
33 extraction, 800 μ l of hexane was added, and mixed for 30 min. Then, 600 μ l of the upper
34
35 layer (hexane) was collected, dried in the Speed-Vac system and dissolved in 150 μ l of
36
37 mobile phase containing acetonitrile:methanol (85:15%, v/v) for stabilization of vitamins. 20
38
39 μ l of this solution was injected into the HPLC system. Standard and control serum samples,
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41 with known α -tocopherol and retinol concentrations, were purchased from Chromsystems and
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43 prepared like the plasma samples.
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51 The HPLC system consisting of a GP 40 gradient pump, GINA 50 autosampler (both
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53 from Dionex) and Jasco FP-920 fluorimetric detector was used for α -tocopherol and retinol
54
55 quantification. Samples were separated in an isocratic system C18 reversed phase column
56
57 Atlantis DC 18 (3 mm x 150 mm x 5 μ m) with guard column. The mobile phase, containing
58
59 acetonitrile and methanol (85:15, v/v), was used at a flow rate of 1.5 ml/min. The effluent was
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3 monitored with fluorimetric detection (ex=340 nm, em=472 nm for retinol and ex=290 nm,
4
5 em=330 nm for α -tocopherol) and analyzed by Dionex Chromeleon software.
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8 For determination of plasma vitamin C and uric acid a standard stock solution (1 mM
9 uric acid) was made by dissolving uric acid in deionized water. Working standards (in the
10 range 10-500 μ M in 5% metaphosphoric acid, MPA) were freshly prepared for each analysis.
11
12 All solutions were carefully protected from light during preparation and analyzes. Standard
13 and control serum samples, with known ascorbic acid concentrations, were purchased from
14 Chromsystems and prepared like the plasma samples. Aliquots (200 μ l) of freshly prepared or
15 freshly thawed plasma samples were mixed with 200 μ l of 20% MPA for protein precipitation
16 and ascorbic acid stabilization. After centrifugation (10 min, 3000 \times g, 4°C), the supernatants
17 were collected and filtered through Millipore microcentrifuge filters (NMWL 5000). Aliquots
18 of 20 μ l from these filtrates were injected into the HPLC system. The HPLC system consisted
19 of HPLC 515 pump and 717 PLUS autosampler (both from Waters), and Photodiode Array
20 Detector 2996 (Waters) was used for ascorbic acid quantification. Samples were separated in
21 the isocratic system C18 reversed-phase column Spherisorb 5 μ m ODS2 250 mm \times 4.6 mm
22 with C18 guard column, at a flow rate of 1 ml/min. The mobile phase containing 5 mM
23 KH_2PO_4 , 1 mM Na_2EDTA , adjusted to pH 3.0 with phosphoric acid, was prepared from
24 deionised water and filtered through a 0.22 μ m membrane before use. The effluent was
25 monitored with a UV detector at 245 nm (ascorbic acid detection), 280 nm (uric acid
26 detection) and analyzed by Empower software.
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51 52 53 54 *Isolation of leukocytes from venous blood*

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57 Blood samples were withdrawn from patients and controls in the morning before breakfast in
58 Clinical Units of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz. Blood
59 samples (18 ml) were carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich
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3 Inc.; St. Louis, MO, USA) and leukocytes were isolated by centrifugation according to the
4
5 manufacturer's procedure.
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10 11 *DNA isolation and 8-oxodG determination in DNA isolates*

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14 DNA from leukocytes was isolated using the method described earlier (35). Briefly; the pellet
15
16 of cells was dispersed by vortexing in ice-cold buffer B (10 mM Tris, 5 mM Na₂EDTA, 0.15
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18 mM deferoxamine mesylate, pH 8.0). A solution of SDS was added (to the final concentration
19
20 of 0.5%), and vortexing was repeated; RNase in 10 mM Tris pH 8.0 was added, and the
21
22 mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the
23
24 mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the
25
26 mixture was gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C
27
28 and transferred to a centrifuge tube containing chloroform/3-methyl-1-butanol and vortexed
29
30 vigorously. After centrifugation, the supernatant containing DNA was treated with 2 volumes
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32 of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate
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34 was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation
35
36 dissolved in nuclease P1 buffer (40 mM sodium acetate, 0.1 mM ZnCl₂, pH 5.1).
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41 Determination of 8-oxodG by means of the HPLC/EC technique was described previously
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43 (36). For genotyping, DNA was extracted from frozen leukocytes using Genomic Mini Kit
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45 (A&A Biotechnology; Gdansk, Poland).
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50 51 *Urine analysis*

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54 Overnight spot urine samples were collected. 0.5 nmol of [¹⁵N₃, ¹³C] 8-oxoGua, 0.05 nmol of
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56 [¹⁵N₅] 8-oxodG and 10 µl of acetic acid (Sigma, HPLC grade) were added to 2 ml of urine.
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59 Isotopic purity of the applied standards was 97.6 and 99.7%, respectively. After centrifugation
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3 (2000×g, 10 min), the supernatant was filtered through a Millipore GV13 0.22 µm syringe
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6 filter, and 500 µl of this solution was injected onto the HPLC system.
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9 Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to
10
11 Gackowski et al. (37).
12

13
14 GC/MS analysis was performed according to the method described by Dizdaroglu
15
16 (38), adapted for additional [¹⁵N₅] 8-oxoGua analyses (m/z 445 and 460 ions were
17
18 monitored).
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21 Cases and controls were interspersed throughout the batches to reduce the possibility
22
23 of batch effects. The samples were run randomly in duplicate. Intrasample coefficient of
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25 variation (cv) for the measurements of 8-oxoGua in urine was 0.9% and intersample
26
27 measurements differed by 6%. Intersample cv for 8-oxodG was 7% and intrasample 3%.
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31 32 33 34 *Preparation of tissue extracts*

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36 Blood leukocytes were homogenized with four volumes of 50 mM Tris-HCl, pH 7.5 buffer
37
38 containing 1 mM EDTA, and proteases inhibitor cocktail (Sigma). Cells were disrupted by
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40 sonication (three 15 s pulses and 30 s intervals), centrifuged (7000×g, 4°C, 30 min), and the
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42 supernatant was collected. Protein concentration was determined by the Bradford method
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44 (39).
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50 51 *8-OxoGua excision activity assay*

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53 8-OxoGua excision activity was measured by the nicking assay (23,40) using ³²P-labelled 40
54
55 nt oligodeoxynucleotide duplex containing a single 8-oxodG, as described previously (10).
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57 The nicking assay allows simultaneous measurements of the glycosylase and AP
58
59 endonuclease activities of the extract. The amount of product was quantified in Molecular
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3 Dynamics Storm 820 PhosphorImager using Image Quant software (Molecular Dynamics,
4 version 5.2). Cases and controls were interspersed throughout the batches to reduce the
5 possibility of batch effects.
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12 *OGG1 genotyping by the Multitemperature PCR-Single Strand Conformation Polymorphism*
13 *(MSSCP) method*
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17 *OGG1* Ser326Cys polymorphism was investigated by the MSSCP method (41). A pair of
18 intron based primers of the sequence: forward 5' ACT GTC ACT AGT CTC ACC AG 3',
19 reverse 5' TGA ATT CGG AAG GTG CTT GGG GAA T 3' (42) was used to PCR amplify
20 exon 7 of the *OGG1* gene. Cycling conditions for *OGG1* PCR-MSSCP were: 95°C for 3 min,
21 35 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C, followed by an extension step of 7
22 min at 72°C. PCR products (4 µl) were denatured and separated by electrophoresis on native
23 10% polyacrylamide gel in 0.5 x TBE buffer in DNA Pointer System Plus (BioVectis,
24 Warsaw, Poland). Initial electrophoresis was performed at 100V for 10 min at 35°C, and
25 subsequently at three temperatures of 35-15-5°C for 30 min each at 40W. DNA bands were
26 visualized by silver staining (BioVectis). Abnormally migrating conformers were sequenced
27 in the Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS.
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46 *RNA extraction and cDNA synthesis*
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48 Total RNA was isolated from frozen leukocytes using the TRIzol reagent (Invitrogen,
49 Carlsbad, CA, USA). The quality of total RNA was checked by formaldehyde-agarose gel
50 electrophoresis, and for further analyses, only RNA samples with clearly distinguished 18S
51 and 28S ribosomal RNAs and no visible RNA degradation were used. Total RNA (1 µg) from
52 each sample was used to generate cDNA using the Advantage RT-for-PCR cDNA synthesis
53 kit (Clontech; Mountain View, CA, USA) with oligo(dT) primers.
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Real-time PCR using SYBR-Green chemistry

Real-time PCR assays were carried out on an Applied Biosystems 7500 apparatus. Each reaction was carried out in 25 μ l mixture containing: 1x Taq polymerase buffer (without $MgCl_2$), 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 5% DMSO, 0.5 ng/ μ l acetylated BSA, dATP, dCTP, dGTP and dTTP – 400 μ M each, 1x concentrated reference dye ROX, 1:40000 diluted SYBR Green, 0.625 U of Taq polymerase, forward and reverse primers, 400 μ M each, and cDNA template. Time-temperature program was as follows: 95°C for 3 min as initial denaturation step followed by 45 cycles consisting of a denaturation step at 95°C for 15 s, primer annealing at 60°C for 15 s and an extension step at 72°C for 1 min. Fluorescence was read during the extension step of each cycle. Melting-point temperature analysis was performed in the range of 60 to 95°C, with temperature increments of 0.33°C. Background range and threshold for C_t evaluation in each experiment were adjusted manually.

The following primers designed using the Primer Express program (Applied Biosystems; Foster City, CA, USA) were used: 5'-ATTCGAACGTCTGCCCTATCA-3' and 5'-TGCCTTCCTTGGATGTGGTAG-3' for the *18S rRNA* gene, 5'-GCCTTTCGCAAGTTCCTGA-3' and 5'-GCGTGAAGCCAGCATTCTTT-3' for *APE1*, 5'-CCCCACGTCTCATGTTG-3' and 5'-CCATCCTTAGCGCTGTCTCC-3' for the *OGG1* gene. Annealing temperatures for these primers were from 58 to 60°C. The product from each pair was 131-132 bp long.

Before use, the primers were tested for equal efficiency of the PCR reaction. To ensure that, the $2^{-\Delta\Delta C_t}$ method validation was applied (43), each experiment involved measurement of C_t values for four or five amounts of the template, each in duplicate. The template amounts per sample were as follows: for *18S rRNA* – 10, 20, 40, 80 and 160 ng, for *APE1* – 40, 80, 160, 320 and 640 ng, for *OGG1* – 80, 160, 320 and 640 ng. The efficiency (which may have a

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2
3 value between 0 and 1) of the QPCR reaction with each primer pair was calculated, and
4
5 subsequently used to calculate the ratio of each studied gene to the reference gene. Only
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7 efficiencies of values ≥ 0.95 were accepted.
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10 For each cDNA sample four reactions were carried out using two template amounts of
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12 10 and 40 ng, each in duplicate. The quality of results was evaluated based on expected C_t
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14 differences between the two cDNA amounts as well as product melting curves. Few rare
15
16 outlying results were omitted in the calculations. For each gene the amounts of cDNA were
17
18 chosen individually (if possible, the same for all genes) to obtain C_t values in the range
19
20 between 14 and 34 cycles.
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24 The results were calculated with normalization of C_t values to mean C_t value for the
25
26 18S rRNA reference gene as described (44).
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31 *Statistical analysis*

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33 Data are presented as median and interquartile range. The statistical differences of the results
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35 were analyzed by Mann-Whitney U test and Mann-Whitney U test with Bonferroni correction
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37 after the Kruskal-Wallis ANOVA. Associations between different variables were calculated
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39 using Spearman's correlation analysis. All statistical analyses were performed using
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41 STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). P values less than 0.05 were considered
42
43 statistically significant.
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50 **Results**

51 *Oxidative status*

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53 In order to investigate the difference in oxidative status between patients with CRC and
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55 adenoma in relation to healthy volunteers, we measured antioxidant vitamins in blood plasma
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57 and the level of oxidized nucleotides in urine of these three groups. CRC patients had
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3 significantly lower levels of ascorbic acid (34.76 μM), α -tocopherol (23.92 μM) and retinol
4 (1.13 μM) as well as uric acid (277.79 μM) in blood plasma than healthy controls (56.70 μM ,
5 35.71 μM , 1.99 μM , and 312.25 μM , $P=0.000000$, 0.00001, 0.00001 and 0.00011,
6 respectively; Table I). In adenoma individuals the level of ascorbic acid (49.79 μM) as well as
7 of uric acid (325.23 μM), which is one of the main ROS scavengers in the human body, was
8 similar to that in control individuals ($P=0.058$ and 0.70, respectively, Table I). The levels of
9 α -tocopherol and retinol in plasma of adenoma individuals (30.15 μM and 1.70 μM ,
10 respectively) were intermediate between those of CRC patients and healthy volunteers, and
11 differences between all groups were statistically significant ($P=0.000000$, C vs. A and 0.0083,
12 A vs. H, for α -tocopherol, and $P=0.000001$, C vs. A and 0.00015, A vs. H for retinol; Table I).
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27 Antioxidant vitamin deficit in individuals, who may be at early or are at later stages of
28 neoplastic transformation (AD and CRC) was accompanied by an increased level of
29 oxidatively damaged DNA. 8-OxodG level in DNA of blood leukocytes was elevated both in
30 CRC patients (6.31 8-oxodG/ 10^6 dG) and AD individuals (5.89 8-oxodG/ 10^6 dG) in relation to
31 healthy volunteers (4.41 8-oxodG/ 10^6 dG, $P=0.000000$, C vs. H and 0.000004, A vs. H, Table
32 II). Urinary excretion of 8-oxoGua was higher for CRC patients than for healthy controls and
33 adenoma individuals (10.07 nmols/mmol creatinine for CRC, vs. 7.68 nmols/mmol creatinine
34 for healthy individuals, $P=0.000075$ and vs. 7.55 nmols/mmol creatinine for adenoma,
35 $P=0.0079$, Table II). In urine of AD individuals the 8-oxoGua level (7.55 nmols/mmol
36 creatinine) was similar to that of healthy volunteers (7.68 nmols/mmol creatinine, $P=0.51$).
37 The level of 8-oxodG was higher in urine of both CRC patients (1.74 nmols/mmol creatinine)
38 and AD individuals (1.70 nmols/mmol creatinine) in comparison to control individuals (1.38
39 nmols/mmol creatinine, $P=0.00026$ and 0.000008, respectively, Table II). There was no
40 correlation between age and antioxidant vitamin levels in colon cancer patients.
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8-OxoGua repair capacity and *OGG1* gene polymorphism

CRC patients revealed significantly higher 8-oxoGua excision activity in leukocytes (40.10 pmols/h/mg protein) than healthy controls (22.50 pmols/h/mg protein, $P=0.000000$; Table III). Due to insufficient quantity of clinical material we were unable to measure 8-oxoGua repair in leukocytes of AD subjects.

Differences in 8-oxoGua repair capacity between CRC patients and controls might be caused by different frequencies of *OGG1* protein Ser326Cys polymorphism in both groups. We identified *OGG1* genotypes of CRC patients and AD individuals, as well as of controls. We found that the population of healthy individuals does not confirm Hardy-Weinberg equilibrium very well. In the healthy control group the frequency of the Ser326Cys genotype is higher and the frequency of Ser326Ser genotype is lower than expected (34% vs. 18% and 65% vs. 81%, respectively; Table IV). In all the studied groups from 35% to 48.7% of individuals carried at least one 326Cys allele. However, among CRC patients there was a significantly higher number of Cys326Cys homozygotes (23%) than in the remaining two groups (1.3% and 1% in AD and controls, respectively, Table IV). This is inconsistent with the expected repair activity distribution among cancer patients and controls in relation to known properties of pure variant proteins (18,29). Although 8-oxoGua excision rate was lower in leukocytes of CRC patients bearing the Cys326Cys genotype (24.5 pmols/h/mg protein) than in those bearing the Ser326Ser genotype (42 pmols/h/mg protein, respectively, Table V), CRC patients with homozygous *OGG1* Cys variant had similar 8-oxoGua repair capacity to that of healthy Ser326Ser control individuals. This may suggest that although *OGG1* polymorphism may have an impact on the rate of 8-oxoGua excision in human tissues, the effective excision activity is also influenced by other factors.

In CRC patients increased levels of 8-oxoGua were observed in urine of both *OGG1* 326 heterozygotes ($P=0.011$) and *OGG1* Ser326Ser homozygotes ($P=0.049$). However, when

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3 comparing 8-oxoGua urinary excretion within the groups of CRC patients and controls, no
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5 significant differences were found in relation to the *OGG1* Ser326Cys polymorphism (Table
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10 The level of 8-oxodG in leukocytes was higher in homozygous Ser/Ser colon cancer
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12 patients than in healthy Ser/Ser individuals ($P=0.000001$). However, heterozygous CRC
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14 patients and heterozygous healthy volunteers had comparable amounts of 8-oxodG in their
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16 leukocytes ($P=0.1$; Table V). When comparing the 8-oxodG level in leukocytes within the
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18 groups of healthy individuals in relation to the *OGG1* polymorphism, no differences were
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20 found between Ser326Ser homozygotes and Ser326Cys heterozygotes. Among CRC patients
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22 the 8-oxodG level in leukocytes was lower in Ser326Cys heterozygotes than in Cys326Cys
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24 and Ser326Ser homozygotes ($P=0.003$ and $P=0.016$, respectively; Table V).

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27 Interestingly, we did not find any correlation between 8-oxoGua repair capacity and 8-
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29 oxodG level in blood leukocytes and urine, either in the group of healthy controls or in CRC
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31 patients, both for the whole groups and the *OGG1* variants.

32 33 34 35 36 37 38 *mRNA level of OGG1, APE1 and MTH1*

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40 In order to obtain an insight into the mechanism of increased 8-oxoGua repair in CRC
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42 patients, we investigated the mRNA level of OGG1 glycosylase and the next enzyme in the
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44 BER pathway – AP endonuclease, APE1, as well as 8-oxodGTP phosphohydrolase, MTH1.
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46 OGG1 and APE1 mRNA levels were about an order of magnitude higher in leukocytes of
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48 CRC patients (1.28 for OGG1 and 88.25 for APE1) and AD individuals (0.99 for OGG1 and
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50 113.83 for APE1) in comparison to healthy volunteers (0.19 for OGG1, $P=0.000000$ C vs. H
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52 and 0.000002 A vs. H, and 13.87 for APE1, $P=0.000000$ C vs. H and 0.000009 A vs. H, Table
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54 VI). There was no difference in OGG1 and APE1 mRNA levels between CRC patients and
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56 AD individuals ($P=0.46$ and 0.5, respectively, Table VI). Also, no statistically significant
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3 differences in mRNA levels of *OGG1* and *APE1* were found among CRC patients and
4 adenoma individuals in relation to the *OGG1* genotype. However, in healthy controls *OGG1*
5 expression in individuals bearing the Ser/Ser genotype was lower than in those with the
6 Ser/Cys genotype ($P=0.008$, Table VI). For *APE1* a strong, but statistically insignificant,
7 tendency of decrease in the *APE1* mRNA level was also observed in healthy volunteers
8 bearing the Ser/Ser rather than the Ser/Cys genotype ($P=0.057$, Table VI). Similarly, the
9 *MTH1* mRNA level was significantly higher in leukocytes of CRC patients (86.82) than of
10 healthy individuals (5.26, $P=0.0012$; Table VI). Interestingly, an increased *MTH1* mRNA
11 level was observed only in CRC patients bearing the *OGG1* 326Cys allele (Table VI).
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25 We found a strong positive correlation between *OGG1* and *APE1* mRNA levels in
26 both healthy controls ($\rho=0.77$, $P=0.000000$, $n=39$) and adenoma individuals and colon cancer
27 patients ($\rho=0.9$, $P=0.000000$, $n=38$ and $\rho=0.68$, $P=0.000000$, $n=45$, respectively). In the
28 group of healthy controls, but not in the group of colon cancer patients and adenoma
29 individuals, there was a positive correlation between *OGG1* and *APE1* mRNA levels and the
30 amount of 8-oxoGua in urine ($\rho=0.41$, $P=0.04$, $n=24$ and $\rho=0.42$, $P=0.04$, $n=24$, respectively).
31 However, we did not observe any association between *OGG1*, *APE1* and *MTH1* mRNA
32 levels and the amount of 8-oxodG in blood leukocyte DNA in all the studied groups.
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46 *Effect of tobacco smoking, sex, age and cancer stage*

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48 No effect of tobacco smoking, sex and age on 8-oxoGua excision rate as well as mRNA level
49 of repair enzymes was observed in leukocytes of CRC patients and controls. Moreover, no
50 differences in leukocyte 8-oxodG level and 8-oxoGua urinary excretion were observed in
51 relation to tumor localization within the colon or rectum. In the majority of adenoma
52 individuals only one polyp was found, and if more were present there was no correlation
53 between measured parameters of oxidative stress and multiplicity of polyps. A weak positive
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3 correlation was found between 8-oxoGua level in urine and the stage of disease ($\rho=0.22$,
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5 $P=0.05$, $n=57$).
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10 Discussion

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12 We measured the oxidative status and 8-oxoGua repair in leukocytes of healthy individuals,
13 patients bearing benign adenomas (AD), and colon cancer (CRC) patients. CRC usually
14 develops by neoplastic transformation of a colon epithelial cell, giving rise to a benign polyp,
15 which subsequently may progress to invasive carcinoma. The group of AD patients might
16 thus represent individuals at an early stage of colon cancer development, and give an insight
17 to the contribution of oxidative processes in colon carcinogenesis in humans. However, it is
18 necessary to bear in mind that probably not all of AD individuals will develop cancer in the
19 future. We used the most extensively applied method of exploring the level of oxidatively
20 damaged DNA in the whole organism, namely determination of 8-oxodG in DNA of
21 surrogate tissues such as white blood cells or urinary excretion of 8-oxoGua and/or 8-oxodG.
22 8-OxoGua may reflect the rate of base excision repair, and 8-oxodG sanitation of the cellular
23 nucleotide pool by the MTH1 directed pathway (12) or less likely nucleotide excision repair
24 (45).
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43 We observed increased oxidative stress in CRC patients, but also in AD individuals in
44 comparison to healthy controls. This manifested as an elevation of the 8-oxodG level in
45 leukocytes and in urine, as well as depletion of antioxidant vitamins in blood plasma.
46 Moreover, increased mRNA levels of repair enzymes, OGG1, APE1 and MTH1 were found
47 in leukocytes of CRC and AD individuals, and an increased 8-oxoGua excision rate in
48 leukocytes of CRC patients with an increased frequency of the *OGG1* Cys326Cys genotype.
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57 An increase in oxidative stress during colon carcinogenesis was demonstrated by several
58 groups. Goodman et al. (46) showed an inverse association between oxidative balance score
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3 (which characterizes pro-oxidant and anti-oxidant exposures) and colorectal adenoma. Leung
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5 et al. (47) further demonstrated that oxidative stress increases during CRC progression from
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7 operable CRC to non-operable liver metastasis, as observed by depletion of antioxidant
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9 vitamins and increase in lipid peroxidation. Several observations also suggest increase in
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11 oxidative processes in cancer tissues (48). The suggested mechanisms responsible for the
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13 oxidative stress in cancer patients include (4): i/ granulocyte activation with release of ROS;
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15 ii/ stimulation of cytokines, of which some, e.g. tumor necrosis factor, produce large amounts
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17 of ROS; iii/ production of hydrogen peroxide by malignant cells, which in advanced stages of
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19 cancer may be released into the blood stream and penetrate into other tissues (2).
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25 The conditions outlined above may not be relevant for patients with benign colon
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27 tumors. However, our results demonstrate that the 8-oxodG level in leukocyte DNA and in
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29 urine is significantly higher in individuals with polyps in comparison with the control group
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31 (Table II). In contrast, urinary excretion of 8-oxoGua, which may reflect excision by DNA
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33 glycosylases, was not elevated in AD individuals (Table II). The nucleotide pool is much
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35 more susceptible to oxidation than dsDNA; hence, 8-oxodG in urine may be a more sensitive
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37 indicator of oxidative processes in the body, and shows that even in AD individuals the
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39 oxidative processes are enhanced. This is confirmed by the observed decrease of antioxidant
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41 vitamins, α -tocopherol and retinol in blood plasma of AD individuals. Further decrease of
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43 these antioxidants, as well as ascorbic acid was observed in CRC patients (Table 1). This may
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45 suggest that antioxidant defense gradually decreases in the course of disease development.
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47 Other studies show a similar depletion of antioxidant vitamins in cancer patients in
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49 comparison to controls (49). The mechanism of such depletion may involve either insufficient
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51 vitamin intake, or malabsorption in the intestine or accelerated usage during disease
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53 progression. Vitamins E and A may also participate in regulation of cell proliferation, e.g., α -
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55 tocopherol inhibits protein kinase C activity and in effect cell proliferation. Supplementation
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3 of animals with vitamin E prevents chemically induced colon cancer (50). Vitamin A
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5 deficiency is responsible for decreased mucosa production and expansion of proliferation
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7 zones (51). Absorption of vitamins C and E occurs *via* intestinal transporters (52,53).
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9 Moreover, cytochrom P450-catalyzed catabolism of tocopherols may be a decisive factor
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11 responsible for the bioavailability of vitamin E (54).
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15 Since in our study the subjects had similar dietary habits, the differences in antioxidant
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17 vitamin levels might be at least partially genetically determined.
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21 The second line of defense against oxidative stress is DNA repair. 8-OxoGua excision
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23 activity was significantly higher in leukocytes of CRC patients in comparison to healthy
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25 volunteers (Table III). The increase in 8-oxoGua repair capacity might be due to increased
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27 transcription from *OGG1* and *APE1* genes, which was observed in AD and CRC individuals
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29 (Table VI). Thus, induction of DNA repair genes seems to occur at an early stage of the
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31 carcinogenic pathway, and may be caused by increased oxidative stress. Expression of *APE1*
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33 and *OGG1* is induced by ROS (26), and we found that oxidative stress is enhanced both in the
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35 CRC and AD group. Our results corroborate the findings of Winnepenninckx and coworkers
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37 (55) that induction of most DNA repair genes occurs very early in the carcinogenic process,
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39 *e.g.*, four years before clinical manifestation of malignant skin melanoma. This may be a part
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41 of a carcinogenic program, which might decrease apoptosis in aberrant cells and/or favor
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43 genomic instability. In yeast and human cells, overexpression of some repair enzymes,
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45 namely methylpurine DNA glycosylase and/or APE1, was associated with frameshift
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47 mutations and microsatellite instability (56).
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54 Increase in 8-oxoGua excision in blood leukocytes of CRC patients was in contrast to
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56 studies of other cancer types, which showed that 8-oxoGua repair capacity was decreased in
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58 lung (23,24) and in head and neck (25) cancer patients. Such a decrease was frequently linked
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60 to the *OGG1* Ser326Cys polymorphism. Pure 326Cys OGG1 variant excises 8-oxoGua and

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3 FapyGua from DNA at lower rates than the wild-type enzyme (28,29). However,
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5 controversial results showing no association between *OGG1* Ser326Cys polymorphism and 8-
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7 oxoGua excision rate have also been reported (26). Recent work of Bravard and coworkers
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9 (57) shows that the *OGG1* Cys variant is more sensitive to oxidative inactivation than the Ser
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11 variant, so different degrees of oxidative stress in different studies might partially explain the
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13 controversies in the literature.
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17 This study shows a higher frequency of Cys326Cys homozygotes among CRC patients
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19 than among AD individuals or the control group (Table IV). Our results in contrast to the
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21 observations of Hansen and coworkers (33), who found a higher frequency of Cys allele
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23 among the controls than CRC patients. Allelic distribution may depend on genetic
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25 background of the local population, specifically if the population is isolated, like it was in
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27 Poland after the Second World War till the nineteen nineties. Another study performed on the
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29 Polish population did not find an association between the *OGG1* polymorphism and CRC
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31 risk, but a higher, although statistically insignificant, frequency of the Ser326Cys genotype
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33 was found in CRC patients in comparison to controls ($P=0.07$) (34). Similarly to our results,
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35 Moreno and coworkers (58) showed an association between *OGG1* Cys/Cys genotype and
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37 CRC risk in the Spanish population. These inconsistent results regarding the association of the
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39 *OGG1* genotype and CRC suggest the influence of other factors.
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46 In our study the effect of the *OGG1* Ser326Cys polymorphism on 8-oxoGua excision
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48 rate was clearly seen in the CRC patient group, in which Cys homozygotes were found to
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50 have a decreased 8-oxoGua excision rate in comparison with Ser homozygotes. Interestingly,
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52 the *OGG1* genotype exerted a limited effect on 8-oxodG level in leukocytes and urinary
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54 excretion of 8-oxoGua (Table V). The only difference found was the decreased level of 8-
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56 oxodG in leukocytes of CRC patients with the *OGG1* Ser326Cys heterozygous genotype in
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58 comparison to both Cys/Cys and Ser/Ser homozygotes. This is difficult to explain, since
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3 leukocytes of CRC patients with at least one Cys allele exhibited an increased mRNA level of
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5 MTH1 phosphohydrolase (Table VI). MTH1 activity prevents incorporation of 8-oxodGTP to
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7 DNA and decreases the level of 8-oxodG in DNA. Thus, several pathways are engaged in
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9 elimination of 8-oxoGua from DNA, for example nucleotide pool sanitation and base excision
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11 repair (10).
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15 In the group of healthy subjects there was significant positive correlation between
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17 mRNA levels of the main enzymes involved in 8-oxoGua removal (OGG1 and APE1), and
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19 the amount of 8-oxoGua in urine. Therefore, our finding is the first experimental evidence
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21 which suggests that urinary 8-oxoGua measurements may be attributed to DNA repair.
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23 However, there was no such correlation in the groups of adenoma individuals and carcinoma
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25 patients. The main reason for this inconsistency may be aberrant DNA oxidation in cancerous
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27 and precancerous conditions which may overshadow the subtle relationship observed in
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29 healthy subjects.
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34 This study shows that oxidative stress and antioxidant vitamin deficiency are increased
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36 in individuals developing colon adenomas and carcinomas, and may suggest that they
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38 contribute to the development of colon cancer. At the early stage of colon carcinogenesis, a
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40 defense pathway for elimination from DNA of 8-oxoGua and FapyGua is induced. This
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42 induction may, nevertheless, be insufficient to counteract the increased DNA damage.
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49
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54 results.
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58 **Author disclosure statement**

No competing financial interests exist.

Abbreviations

8-oxodG - 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua – 8-oxo-7,8-dihydroguanine; AD – adenoma; CRC – colorectal cancer; cv – coefficient of variation; FapyGua – 2,6-diamino-4-hydroxy-5-formamidopyrimidine; MPA – metaphosphoric acid; MSSCP - Multitemperature PCR-Single Strand Conformation Polymorphism; ROS – reactive oxygen species; SDS – sodium dodecyl sulfate

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Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients

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Running title: Oxidative stress and 8-oxoGua repair in CRC patients

Key words: 8-oxoguanine, DNA repair, colon cancer, polyps, antioxidants

Abstract

Oxidative stress is involved in the pathogenesis of colon cancer. We wanted to elucidate at which stage of the disease **this phenomenon occurs**. In the examined groups of **patients with** colorectal carcinoma (CRC, n=89), benign adenoma (AD, n=77), and healthy volunteers (controls, n=99) we measured: vitamins A, C and E in blood plasma, 8-oxodG and 8-oxoGua in leukocytes and urine, leukocyte 8-oxoGua excision activity, mRNA levels of APE1, OGG1, MTH1 and *OGGI* polymorphism. **The** vitamin levels decreased gradually in AD and CRC patients. 8-OxodG increased in leukocytes and urine of CRC and AD patients. 8-OxoGua was higher only in **the** urine of CRC patients. 8-OxoGua excision was higher in CRC patients than in controls, in spite of higher frequency of **the** *OGGI* Cys326Cys genotype, encoding **a** glycosylase **with** decreased activity. mRNA levels of OGG1 and APE1 increased in CRC and AD patients, which could explain increased 8-oxoGua excision rate in CRC patients. MTH1 mRNA was also higher in CRC patients. **The** results suggest **that** oxidative stress **occurs** in CRC and AD individuals. This is accompanied by increased transcription of DNA repair genes, and increased 8-oxoGua excision rate in CRC patients, which is, however, insufficient to counteract the increased DNA damage.

Introduction

Colorectal cancer (CRC) is one of the most frequent causes of death in western countries. The most important etiological factors of sporadic colorectal tumors are inflammation, fat metabolism, tobacco smoking as well as consumption of meat and alcohol (1). Inflammation is associated with the release of large amounts of reactive oxygen species (ROS) (2), leading to oxidation of nucleic acids, proteins and lipids. Many epidemiological studies report an inverse association between vegetable and fruit consumption and occurrence of colon cancer (3). One of the possible mechanisms of the protective effect of such food constituents as vitamins A, C and E is by exerting antioxidative activities, by scavenging free radicals and preventing DNA damage. Oxidatively damaged DNA has been blamed for the physiological changes associated with degenerative diseases such as cancer (4,5). A plethora of damaged DNA bases are formed upon ROS attack on genetic material, several of them reveal strong promutagenic properties (6). One of the major and best studied is 8-oxo-7,8-dihydroguanine (8-oxoGua), a typical biomarker of oxidative stress, which may play a role in carcinogenesis (7). The presence of 8-oxo-7,8-dihydroguanosine (8-oxodG) residues in DNA leads to GC→TA transversions (8). 8-OxodG is formed in DNA either *via* direct oxidation of nucleic acids or can be incorporated from the nucleotide pool by DNA polymerases, the latter process being an important source of DNA oxidation and genome instability (9,10). Incorporation of 8-oxodGTP into DNA by DNA polymerases is limited by the activity of MTH1 phosphohydrolase, which hydrolyzes 8-oxodGTP to 8-oxodGMP (11). 8-OxodGMP is subsequently dephosphorylated by nucleotidase and removed from the cell (12). Many observations indicate a direct correlation between 8-oxodG formation and carcinogenesis *in vivo* (7,13-16).

To counteract the deleterious effect of oxidatively damaged DNA, all organisms have developed several DNA repair pathways. Excision of 8-oxoGua from DNA is accomplished

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2
3 mainly by base excision repair. Several DNA glycosylases, which specifically recognize and
4 remove 8-oxoGua in human cells have been described, the major one apparently being OGG1
5 DNA glycosylase (17). Human OGG1 glycosylase recognizes and excises several lesions
6 from oligodeoxynucleotides with single DNA damages, including 8-oxoGua, FapyGua, Me-
7 FapyGua, 8-oxoAde (18,19). The murine enzyme was also shown to excise 8-oxo-inosine,
8 O⁶-methyl-8-oxoGua and 8-amino-Gua (20). However, when γ -irradiated DNA was used as a
9 substrate, pure human OGG1 liberated only 8-oxoGua and FapyGua, as measured by
10 GC/IDMS (18). FapyGua, but not 8-oxoGua, are also eliminated from DNA by NEIL1 DNA
11 glycosylase (21). Both these DNA lesions show strong mutagenic activity in mammalian cells
12 (22). Numerous experimental data suggest a decrease in DNA repair efficiency in cancer
13 patients (23-27). One possibility is that such a decrease may be caused by the presence of
14 polymorphic forms of DNA repair genes, which encode proteins with reduced enzymatic
15 activities (26). Several polymorphic changes in the *OGG1* gene have been described, with the
16 most common being Ser326Cys (28). Polymorphic Cys326 OGG1 protein was found to have
17 a lower enzymatic activity, both when 8-oxoGua was excised from oligodeoxynucleotides
18 (29), and when 8-oxoGua and FapyGua were liberated from γ -irradiated DNA by pure
19 Cys326 or Ser326 OGG1 enzyme (18,28). It was suggested that the presence of two *OGG1*
20 326Cys alleles may confer an increased risk of lung, prostate and nasopharyngeal cancer (30-
21 32), but no association with the risk for colon cancer (33,34). Our and other functional studies
22 report a decrease in 8-oxoGua excision rate in lung (23,24) and head and neck cancer patients
23 (25). Such a decrease in repair rate and simultaneous increase in 8-oxodG and FapydG levels
24 in cellular DNA favors a pro-oxidant state and may accelerate the acquisition of mutations in
25 critical genes leading to cancer. Such an idea is basically derived from the observation that a
26 pro-oxidant environment is characteristic for advanced stages of cancer. However, it is not
27 clear whether increased oxidative stress/oxidatively damaged DNA is merely the result of the

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3 disease or whether it plays a role in cancer development. This prompted us to investigate the
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5 broad range of biomarkers: oxidatively damaged DNA, the status of antioxidant vitamins, and
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8 the repair of 8-oxoGua in colon cancer patients, in individuals developing benign
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10 adenomatous polyps and in healthy controls. We observed an increase in oxidatively damaged
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12 DNA and decreased antioxidant defense in leukocytes of colon cancer patients but also in
13
14 adenoma individuals, of which some may be at very early stages of CRC development.
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16 Increased oxidative stress stimulates the 8-oxoGua excision rate, although this stimulation is
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18 insufficient to counteract oxidative damage to DNA.
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27 **Materials and methods**

28 *Materials*

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30 T4 polynucleotide kinase and [γ -³²P]ATP were from GE Healthcare. Oligodeoxynucleotide
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32 (40 nt) containing a single 8-oxodG at position 20 in the sequence 5'-d(GCT ACC TAC CTA
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34 GCG ACC TXC GAC TGT CCC ACT GCT CGA A)-3', where X indicates 8-oxodG was
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36 obtained from Eurogentec Herstal, (Herstal, Belgium). A complementary
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38 oligodeoxynucleotide containing dC opposite 8-oxodG was synthesized in the
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43 Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS (Warsaw, Poland).
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48 *Study group*

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51 This case-control study was conducted in three groups. The control group consisted of 99
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53 healthy individuals (44 male and 55 female) of median age 55 years (range 42 to 65 years).
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55 The polyp individuals group (AD, n=77, 28 serrated adenomas and 49 adenomas) comprised
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57 38 females and 39 males with a median age of 60 years (range 32 to 83). The colorectal
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59 cancer patient group (CRC, n=89) comprised 45 males and 44 females (median age 62 years,
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3 range 27 to 90). All participants were Caucasians and there were no relatives among them. All
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5 individuals participating in the study were recruited through the hospital (**Collegium**
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7 **Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland**) and were examined by
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9 colonoscopy. **The** control group was recruited from individuals undergoing routine colon
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11 cancer screening, in whom colonoscopy revealed no cancer or polyps. All the subjects, when
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13 recruited to the study, filled in a questionnaire concerning demographic data, smoking, diet
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15 and medical history. Interviewees were asked to estimate the average frequency of
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17 consumption of various dietary items in the year preceding the interview. The majority of
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19 them reportedly consumed 3 servings of fruit and vegetables and about 250 g of meat and fat
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21 per day. To make the group even more homogenous, the subjects who reported the extreme
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23 consumption, as well as those who reported supplementation within the last month were
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25 excluded from the study. The questionnaire was administered by the team physician (Dr.
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27 Banaszkiwicz). The control group was chosen to maximally match the patient groups and
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29 adenoma individuals by age, sex, diet (consumption of fat, carbohydrates and vitamin intake),
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31 body weight, and smoking status. Among healthy volunteers, AD individuals and CRC
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33 patients, two groups were distinguished in relation to their smoking status, namely **those who**
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35 **had never smoked** and smokers who consumed 20 or more cigarettes per day.
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44 Blood and urine was drawn from CRC and AD patients at diagnosis, and from control
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46 individuals, **when** they were recruited to the study following colonoscopy verifying they were
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48 free of any morphological changes within the colon and rectum. Diagnoses of all polyps and
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50 adenocarcinomas were confirmed by histological examination. All the CRC patients had
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52 histologically proven adenocarcinomas at: A (n=4), B1 (n=25), B2 (n=13), C1 (n=3), C2
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54 (n=27) and D (n=17) stage according to Duke's scale with Astler-Coller modification. In 57
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56 cases, cancer developed in the colon, and in 20 patients, in the rectum. In the majority of
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58 cases only one tumor was identified. The information concerning tumor histology and patient
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3 questionnaire responses were blinded to all investigators (with the exception of the team
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5 physician) until after the statistical analysis was completed.
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9 The patients were not treated with any anticancer drugs or vitamins during the time
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11 from the diagnoses until surgery (up to 4 weeks).
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14 The study was conducted in accordance with the Helsinki Declaration and was
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16 approved by the medical ethics committee of Collegium Medicum, Nicolaus Copernicus
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18 University, Bydgoszcz, Poland. All participants signed informed consent.
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21 22 23 *Determination of plasma vitamins A, E, C and uric acid concentration by HPLC*

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25 Quantification of vitamin E (α -tocopherol), vitamin A (retinol), vitamin C (ascorbic acid) and
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27 uric acid by HPLC technique was as previously described (35). Briefly, for vitamin A and E
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29 measurement, freshly prepared or freshly thawed plasma samples were mixed with 200 μ l of
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31 HPLC-grade water and 400 μ l of ethanol in order to precipitate proteins. For vitamin
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33 extraction, 800 μ l of hexane was added, and mixed for 30 min. Then, 600 μ l of the upper
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35 layer (hexane) was collected, dried in the Speed-Vac system and dissolved in 150 μ l of
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37 mobile phase containing acetonitrile:methanol (85:15%, v/v) for stabilization of vitamins. 20
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39 μ l of this solution was injected into the HPLC system. Standard and control serum samples,
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41 with known α -tocopherol and retinol concentrations, were purchased from Chromsystems and
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43 prepared like the plasma samples.
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51 The HPLC system consisting of a GP 40 gradient pump, GINA 50 autosampler (both
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53 from Dionex) and Jasco FP-920 fluorimetric detector was used for α -tocopherol and retinol
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55 quantification. Samples were separated in an isocratic system C18 reversed phase column
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57 Atlantis DC 18 (3 mm x 150 mm x 5 μ m) with guard column. The mobile phase, containing
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59 acetonitrile and methanol (85:15, v/v), was used at a flow rate of 1.5 ml/min. The effluent was
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3 monitored with fluorimetric detection (ex=340 nm, em=472 nm for retinol and ex=290 nm,
4 em=330 nm for α -tocopherol) and analyzed by Dionex Chromeleon software.
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8 For determination of plasma vitamin C and uric acid a standard stock solution (1 mM
9 uric acid) was made by dissolving uric acid in deionized water. Working standards (in the
10 range 10-500 μ M in 5% metaphosphoric acid, MPA) were freshly prepared for each analysis.
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12 All solutions were carefully protected from light during preparation and analyzes. Standard
13 and control serum samples, with known ascorbic acid concentrations, were purchased from
14 Chromsystems and prepared like the plasma samples. Aliquots (200 μ l) of freshly prepared or
15 freshly thawed plasma samples were mixed with 200 μ l of 20% MPA for protein precipitation
16 and ascorbic acid stabilization. After centrifugation (10 min, 3000 \times g, 4°C), the supernatants
17 were collected and filtered through Millipore microcentrifuge filters (NMWL 5000). Aliquots
18 of 20 μ l from these filtrates were injected into the HPLC system. The HPLC system consisted
19 of HPLC 515 pump and 717 PLUS autosampler (both from Waters), and Photodiode Array
20 Detector 2996 (Waters) was used for ascorbic acid quantification. Samples were separated in
21 the isocratic system C18 reversed-phase column Spherisorb 5 μ m ODS2 250 mm \times 4.6 mm
22 with C18 guard column, at a flow rate of 1 ml/min. The mobile phase containing 5 mM
23 KH_2PO_4 , 1 mM Na_2EDTA , adjusted to pH 3.0 with phosphoric acid, was prepared from
24 deionised water and filtered through a 0.22 μ m membrane before use. The effluent was
25 monitored with a UV detector at 245 nm (ascorbic acid detection), 280 nm (uric acid
26 detection) and analyzed by Empower software.
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54 *Isolation of leukocytes from venous blood*

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57 Blood samples were withdrawn from patients and controls in the morning before breakfast in
58 Clinical Units of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz. Blood
59 samples (18 ml) were carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich
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3 Inc.; St. Louis, MO, USA) and leukocytes were isolated by centrifugation according to the
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5 manufacturer's procedure.
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10 11 *DNA isolation and 8-oxodG determination in DNA isolates*

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14 DNA from leukocytes was isolated using the method described earlier (35). Briefly; the pellet
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16 of cells was dispersed by vortexing in ice-cold buffer B (10 mM Tris, 5 mM Na₂EDTA, 0.15
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18 mM deferoxamine mesylate, pH 8.0). A solution of SDS was added (to the final concentration
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20 of 0.5%), and vortexing was repeated; RNase in 10 mM Tris pH 8.0 was added, and the
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22 mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the
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24 mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the
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26 mixture was gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C
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28 and transferred to a centrifuge tube containing chloroform/3-methyl-1-butanol and vortexed
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30 vigorously. After centrifugation, the supernatant containing DNA was treated with 2 volumes
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32 of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate
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34 was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation
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36 dissolved in nuclease P1 buffer (40 mM sodium acetate, 0.1 mM ZnCl₂, pH 5.1).
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41 Determination of 8-oxodG by means of the HPLC/EC technique was described previously
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43 (36). For genotyping, DNA was extracted from frozen leukocytes using Genomic Mini Kit
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45 (A&A Biotechnology; Gdansk, Poland).
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50 51 *Urine analysis*

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54 Overnight spot urine samples were collected. 0.5 nmol of [¹⁵N₃, ¹³C] 8-oxoGua, 0.05 nmol of
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56 [¹⁵N₅] 8-oxodG and 10 µl of acetic acid (Sigma, HPLC grade) were added to 2 ml of urine.
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58
59 Isotopic purity of the applied standards was 97.6 and 99.7%, respectively. After centrifugation
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3 (2000×g, 10 min), the supernatant was filtered through a Millipore GV13 0.22 µm syringe
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5
6 filter, and 500 µl of this solution was injected onto the HPLC system.
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8
9 Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to
10
11 Gackowski et al. (37).
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14 GC/MS analysis was performed according to the method described by Dizdaroglu
15
16 (38), adapted for additional [¹⁵N₅] 8-oxoGua analyses (m/z 445 and 460 ions were
17
18 monitored).
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20
21 Cases and controls were interspersed throughout the batches to reduce the possibility
22
23 of batch effects. The samples were run randomly in duplicate. Intrasample coefficient of
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25 variation (cv) for the measurements of 8-oxoGua in urine was 0.9% and intersample
26
27 measurements differed by 6%. Intersample cv for 8-oxodG was 7% and intrasample 3%.
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31 32 33 *Preparation of tissue extracts*

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35 Blood leukocytes were homogenized with four volumes of 50 mM Tris-HCl, pH 7.5 buffer
36
37 containing 1 mM EDTA, and proteases inhibitor cocktail (Sigma). Cells were disrupted by
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39 sonication (three 15 s pulses and 30 s intervals), centrifuged (7000×g, 4°C, 30 min), and the
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41 supernatant was collected. Protein concentration was determined by the Bradford method
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43 (39).
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50 51 *8-OxoGua excision activity assay*

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53 8-OxoGua excision activity was measured by the nicking assay (23,40) using ³²P-labelled 40
54
55 nt oligodeoxynucleotide duplex containing a single 8-oxodG, as described previously (10).
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57 The nicking assay allows simultaneous measurements of the glycosylase and AP
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59 endonuclease activities of the extract. The amount of product was quantified in Molecular
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3 Dynamics Storm 820 PhosphorImager using Image Quant software (Molecular Dynamics,
4 version 5.2). Cases and controls were interspersed throughout the batches to reduce the
5 possibility of batch effects.
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12 *OGG1* genotyping by *the* Multitemperature PCR-Single Strand Conformation Polymorphism
13 (*MSSCP*) method
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17 *OGG1* Ser326Cys polymorphism was investigated by the MSSCP method (41). A pair of
18 intron based primers of the sequence: forward 5' ACT GTC ACT AGT CTC ACC AG 3',
19 reverse 5' TGA ATT CGG AAG GTG CTT GGG GAA T 3' (42) was used to PCR amplify
20 exon 7 of the *OGG1* gene. Cycling conditions for *OGG1* PCR-MSSCP were: 95°C for 3 min,
21 35 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C, followed by an extension step of 7
22 min at 72°C. PCR products (4 µl) were denatured and separated by electrophoresis on native
23 10% polyacrylamide gel in 0.5 x TBE buffer in DNA Pointer System Plus (BioVectis,
24 Warsaw, Poland). Initial electrophoresis was performed at 100V for 10 min at 35°C, and
25 subsequently at three temperatures of 35-15-5°C for 30 min each at 40W. DNA bands were
26 visualized by silver staining (BioVectis). Abnormally migrating conformers were sequenced
27 in *the* Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS.
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46 *RNA extraction and cDNA synthesis*
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48 Total RNA was isolated from frozen leukocytes using the TRIzol reagent (Invitrogen,
49 Carlsbad, CA, USA). The quality of total RNA was checked by formaldehyde-agarose gel
50 electrophoresis, and for further analyses, only RNA samples with clearly distinguished 18S
51 and 28S ribosomal RNAs and no visible RNA *degradation* were used. Total RNA (1 µg) from
52 each sample was used to generate cDNA using the Advantage RT-for-PCR cDNA synthesis
53 kit (Clontech; Mountain View, CA, USA) with oligo(dT) primers.
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Real-time PCR using SYBR-Green chemistry

Real-time PCR assays were carried out on an Applied Biosystems 7500 apparatus. Each reaction was carried out in 25 μ l mixture containing: 1x Taq polymerase buffer (without $MgCl_2$), 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 5% DMSO, 0.5 ng/ μ l acetylated BSA, dATP, dCTP, dGTP and dTTP – 400 μ M each, 1x concentrated reference dye ROX, 1:40000 diluted SYBR Green, 0.625 U of Taq polymerase, forward and reverse primers, 400 μ M each, and cDNA template. Time-temperature program was as follows: 95°C for 3 min as initial denaturation step followed by 45 cycles consisting of a denaturation step at 95°C for 15 s, primer annealing at 60°C for 15 s and an extension step at 72°C for 1 min. Fluorescence was read during the extension step of each cycle. Melting-point temperature analysis was performed in the range of 60 to 95°C, with temperature increments of 0.33°C. Background range and threshold for C_t evaluation in each experiment were adjusted manually.

The following primers designed using the Primer Express program (Applied Biosystems; Foster City, CA, USA) were used: 5'-ATTCGAACGTCTGCCCTATCA-3' and 5'-TGCCTTCCTTGGATGTGGTAG-3' for the *18S rRNA* gene, 5'-GCCTTTCGCAAGTTCCTGA-3' and 5'-GCGTGAAGCCAGCATTCTTT-3' for *APE1*, 5'-CCCCACGTCTCATGTTG-3' and 5'-CCATCCTTAGCGCTGTCTCC-3' for the *OGGI* gene. **Annealing temperatures** for these primers were from 58 to 60°C. The product from each pair was 131-132 bp long.

Before use, the primers were **tested** for equal efficiency of the PCR reaction. To ensure that, the $2^{-\Delta\Delta C_t}$ method validation was applied (43), each experiment involved measurement of C_t values for four or five amounts of the template, each in duplicate. The template amounts per sample were as follows: for *18S rRNA* – 10, 20, 40, 80 and 160 ng, for *APE1* – 40, 80, 160, 320 and 640 ng, for *OGGI* – 80, 160, 320 and 640 ng. The efficiency (which may have a

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2
3 value between 0 and 1) of the QPCR reaction with each primer pair was calculated, and
4
5 subsequently used to calculate the ratio of each studied gene to the reference gene. Only
6
7 efficiencies of values ≥ 0.95 were accepted.
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10 For each cDNA sample four reactions were carried out using two template amounts of
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12 10 and 40 ng, each in duplicate. The quality of results was evaluated based on expected C_t
13
14 differences between the two cDNA amounts as well as product melting curves. Few rare
15
16 outlying results were omitted in the calculations. For each gene the amounts of cDNA were
17
18 chosen individually (if possible, the same for all genes) to obtain C_t values in the range
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20 between 14 and 34 cycles.
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24 The results were calculated with normalization of C_t values to mean C_t value for the
25
26 18S rRNA reference gene as described (44).
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31 *Statistical analysis*

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33 Data are presented as median and interquartile range. The statistical differences of the results
34
35 were analyzed by Mann-Whitney U test and Mann-Whitney U test with Bonferroni correction
36
37 after the Kruskal-Wallis ANOVA. Associations between different variables were calculated
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39 using Spearman's correlation analysis. All statistical analyses were performed using
40
41 STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). P values less than 0.05 were considered
42
43 statistically significant.
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50 **Results**

51 *Oxidative status*

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53 In order to investigate the difference in oxidative status between patients with CRC and
54
55 adenoma in relation to healthy volunteers, we measured antioxidant vitamins in blood plasma
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57 and the level of oxidized nucleotides in urine of these three groups. CRC patients had
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3 significantly lower levels of ascorbic acid (34.76 μM), α -tocopherol (23.92 μM) and retinol
4 (1.13 μM) as well as uric acid (277.79 μM) in blood plasma than healthy controls (56.70 μM ,
5 35.71 μM , 1.99 μM , and 312.25 μM , $P=0.000000$, 0.00001, 0.00001 and 0.00011,
6 respectively; Table I). In adenoma individuals the level of ascorbic acid (49.79 μM) as well as
7 of uric acid (325.23 μM), which is one of the main ROS scavengers in the human body, was
8 similar to that in control individuals ($P=0.058$ and 0.70, respectively, Table I). The levels of
9 α -tocopherol and retinol in plasma of adenoma individuals (30.15 μM and 1.70 μM ,
10 respectively) were intermediate between those of CRC patients and healthy volunteers, and
11 differences between all groups were statistically significant ($P=0.000000$, C vs. A and 0.0083,
12 A vs. H, for α -tocopherol, and $P=0.00001$, C vs. A and 0.00015, A vs. H for retinol; Table I).

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Antioxidant vitamin deficit in individuals, who may be at early or are at later stages of neoplastic transformation (AD and CRC) was accompanied by an increased level of oxidatively damaged DNA. 8-OxodG level in DNA of blood leukocytes was elevated both in CRC patients (6.31 8-oxodG/10⁶dG) and AD individuals (5.89 8-oxodG/10⁶dG) in relation to healthy volunteers (4.41 8-oxodG/10⁶dG, $P=0.000000$, C vs. H and 0.000004, A vs. H, Table II). Urinary excretion of 8-oxoGua was higher for CRC patients than for healthy controls and adenoma individuals (10.07 nmols/mmol creatinine for CRC, vs. 7.68 nmols/mmol creatinine for healthy individuals, $P=0.000075$ and vs. 7.55 nmols/mmol creatinine for adenoma, $P=0.0079$, Table II). In urine of AD individuals the 8-oxoGua level (7.55 nmols/mmol creatinine) was similar to that of healthy volunteers (7.68 nmols/mmol creatinine, $P=0.51$). The level of 8-oxodG was higher in urine of both CRC patients (1.74 nmols/mmol creatinine) and AD individuals (1.70 nmols/mmol creatinine) in comparison to control individuals (1.38 nmols/mmol creatinine, $P=0.00026$ and 0.000008, respectively, Table II). There was no correlation between age and antioxidant vitamin levels in colon cancer patients.

8-OxoGua repair capacity and OGG1 gene polymorphism

CRC patients revealed significantly higher 8-oxoGua excision activity in leukocytes (40.10 pmols/h/mg protein) than healthy controls (22.50 pmols/h/mg protein, $P=0.000000$; Table III). Due to insufficient quantity of clinical material we were unable to measure 8-oxoGua repair in leukocytes of AD subjects.

Differences in 8-oxoGua repair capacity between CRC patients and controls might be caused by different frequencies of OGG1 protein Ser326Cys polymorphism in both groups. We identified *OGG1* genotypes of CRC patients and AD individuals, as well as of controls. We found that the population of healthy individuals does not confirm Hardy-Weinberg equilibrium very well. In the healthy control group the frequency of the Ser326Cys genotype is higher and the frequency of Ser326Ser genotype is lower than expected (34% vs. 18% and 65% vs. 81%, respectively; Table IV). In all the studied groups from 35% to 48.7% of individuals carried at least one 326Cys allele. However, among CRC patients there was a significantly higher number of Cys326Cys homozygotes (23%) than in the remaining two groups (1.3% and 1% in AD and controls, respectively, Table IV). This is inconsistent with the expected repair activity distribution among cancer patients and controls in relation to known properties of pure variant proteins (18,29). Although 8-oxoGua excision rate was lower in leukocytes of CRC patients bearing the Cys326Cys genotype (24.5 pmols/h/mg protein) than in those bearing the Ser326Ser genotype (42 pmols/h/mg protein, respectively, Table V), CRC patients with homozygous *OGG1* Cys variant had similar 8-oxoGua repair capacity to that of healthy Ser326Ser control individuals. This may suggest that although *OGG1* polymorphism may have an impact on the rate of 8-oxoGua excision in human tissues, the effective excision activity is also influenced by other factors.

In CRC patients increased levels of 8-oxoGua were observed in urine of both *OGG1* 326 heterozygotes ($P=0.011$) and *OGG1* Ser326Ser homozygotes ($P=0.049$). However, when

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3 comparing 8-oxoGua urinary excretion within the groups of CRC patients and controls, no
4
5 significant differences were found in relation to the *OGG1* Ser326Cys polymorphism (Table
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7 V).

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10 The level of 8-oxodG in leukocytes was higher in homozygous Ser/Ser colon cancer
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12 patients than in healthy Ser/Ser individuals ($P=0.000001$). However, heterozygous CRC
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14 patients and heterozygous healthy volunteers had comparable amounts of 8-oxodG in their
15
16 leukocytes ($P=0.1$; Table V). When comparing the 8-oxodG level in leukocytes within the
17
18 groups of healthy individuals in relation to the *OGG1* polymorphism, no differences were
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20 found between Ser326Ser homozygotes and Ser326Cys heterozygotes. Among CRC patients
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22 the 8-oxodG level in leukocytes was lower in Ser326Cys heterozygotes than in Cys326Cys
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24 and Ser326Ser homozygotes ($P=0.003$ and $P=0.016$, respectively; Table V).

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27 Interestingly, we did not find any correlation between 8-oxoGua repair capacity and 8-
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29 oxodG level in blood leukocytes and urine, either in the group of healthy controls or in CRC
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31 patients, both for the whole groups and the *OGG1* variants.

32 33 34 35 36 37 38 *mRNA level of OGG1, APE1 and MTH1*

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40 In order to obtain an insight into the mechanism of increased 8-oxoGua repair in CRC
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42 patients, we investigated the mRNA level of OGG1 glycosylase and the next enzyme in the
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44 BER pathway – AP endonuclease, APE1, as well as 8-oxodGTP phosphohydrolase, MTH1.
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46 OGG1 and APE1 mRNA levels were about an order of magnitude higher in leukocytes of
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48 CRC patients (1.28 for OGG1 and 88.25 for APE1) and AD individuals (0.99 for OGG1 and
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50 113.83 for APE1) in comparison to healthy volunteers (0.19 for OGG1, $P=0.000000$ C vs. H
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52 and 0.000002 A vs. H, and 13.87 for APE1, $P=0.000000$ C vs. H and 0.000009 A vs. H, Table
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54 VI). There was no difference in OGG1 and APE1 mRNA levels between CRC patients and
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56 AD individuals ($P=0.46$ and 0.5, respectively, Table VI). Also, no statistically significant
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3 differences in mRNA levels of *OGG1* and *APE1* were found among CRC patients and
4 adenoma individuals in relation to the *OGG1* genotype. However, in healthy controls *OGG1*
5 expression in individuals bearing the Ser/Ser genotype was lower than in those with the
6 Ser/Cys genotype ($P=0.008$, Table VI). For *APE1* a strong, but statistically insignificant,
7 tendency of decrease in the *APE1* mRNA level was also observed in healthy volunteers
8 bearing the Ser/Ser rather than the Ser/Cys genotype ($P=0.057$, Table VI). Similarly, the
9 *MTH1* mRNA level was significantly higher in leukocytes of CRC patients (86.82) than of
10 healthy individuals (5.26, $P=0.0012$; Table VI). Interestingly, an increased *MTH1* mRNA
11 level was observed only in CRC patients bearing the *OGG1* 326Cys allele (Table VI).
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25 We found a strong positive correlation between *OGG1* and *APE1* mRNA levels in
26 both healthy controls ($\rho=0.77$, $P=0.000000$, $n=39$) and adenoma individuals and colon cancer
27 patients ($\rho=0.9$, $P=0.000000$, $n=38$ and $\rho=0.68$, $P=0.000000$, $n=45$, respectively). In the
28 group of healthy controls, but not in the group of colon cancer patients and adenoma
29 individuals, there was a positive correlation between *OGG1* and *APE1* mRNA levels and the
30 amount of 8-oxoGua in urine ($\rho=0.41$, $P=0.04$, $n=24$ and $\rho=0.42$, $P=0.04$, $n=24$, respectively).
31 However, we did not observe any association between *OGG1*, *APE1* and *MTH1* mRNA
32 levels and the amount of 8-oxodG in blood leukocyte DNA in all the studied groups.
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46 *Effect of tobacco smoking, sex, age and cancer stage*

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48 No effect of tobacco smoking, sex and age on 8-oxoGua excision rate as well as mRNA level
49 of repair enzymes was observed in leukocytes of CRC patients and controls. Moreover, no
50 differences in leukocyte 8-oxodG level and 8-oxoGua urinary excretion were observed in
51 relation to tumor localization within the colon or rectum. In the majority of adenoma
52 individuals only one polyp was found, and if more were present there was no correlation
53 between measured parameters of oxidative stress and multiplicity of polyps. A weak positive
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3 correlation was found between 8-oxoGua level in urine and the stage of disease ($p=0.22$,
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5 $P=0.05$, $n=57$).
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10 Discussion

11 We measured the oxidative status and 8-oxoGua repair in leukocytes of healthy individuals,
12 patients bearing benign adenomas (AD), and colon cancer (CRC) patients. CRC usually
13 develops by neoplastic transformation of a colon epithelial cell, giving rise to a benign polyp,
14 which subsequently may progress to invasive carcinoma. The group of AD patients might
15 thus represent individuals at an early stage of colon cancer development, and give an insight
16 to the contribution of oxidative processes in colon carcinogenesis in humans. However, it is
17 necessary to bear in mind that probably not all of AD individuals will develop cancer in the
18 future. We used the most extensively applied method of exploring the level of oxidatively
19 damaged DNA in the whole organism, namely determination of 8-oxodG in DNA of
20 surrogate tissues such as white blood cells or urinary excretion of 8-oxoGua and/or 8-oxodG.
21 8-OxoGua may reflect the rate of base excision repair, and 8-oxodG sanitation of the cellular
22 nucleotide pool by the MTH1 directed pathway (12) or less likely nucleotide excision repair
23 (45).
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43 We observed increased oxidative stress in CRC patients, but also in AD individuals in
44 comparison to healthy controls. This manifested as an elevation of the 8-oxodG level in
45 leukocytes and in urine, as well as depletion of antioxidant vitamins in blood plasma.
46 Moreover, increased mRNA levels of repair enzymes, OGG1, APE1 and MTH1 were found
47 in leukocytes of CRC and AD individuals, and an increased 8-oxoGua excision rate in
48 leukocytes of CRC patients with an increased frequency of the *OGG1* Cys326Cys genotype.
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57 An increase in oxidative stress during colon carcinogenesis was demonstrated by several
58 groups. Goodman et al. (46) showed an inverse association between oxidative balance score
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3 (which characterizes pro-oxidant and anti-oxidant exposures) and colorectal adenoma. Leung
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5 et al. (47) further demonstrated that oxidative stress increases during CRC progression from
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7 operable CRC to non-operable liver metastasis, as observed by depletion of antioxidant
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9 vitamins and increase in lipid peroxidation. Several observations also suggest increase in
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11 oxidative processes in cancer tissues (48). The suggested mechanisms responsible for the
12
13 oxidative stress in cancer patients include (4): i/ granulocyte activation with release of ROS;
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15 ii/ stimulation of cytokines, of which some, e.g. tumor necrosis factor, produce large amounts
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17 of ROS; iii/ production of hydrogen peroxide by malignant cells, which in advanced stages of
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19 cancer may be released into the blood stream and penetrate into other tissues (2).
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25 The conditions outlined above may not be relevant for patients with benign colon
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27 tumors. However, our results demonstrate that the 8-oxodG level in leukocyte DNA and in
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29 urine is significantly higher in individuals with polyps in comparison with the control group
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31 (Table II). In contrast, urinary excretion of 8-oxoGua, which may reflect excision by DNA
32
33 glycosylases, was not elevated in AD individuals (Table II). The nucleotide pool is much
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35 more susceptible to oxidation than dsDNA; hence, 8-oxodG in urine may be a more sensitive
36
37 indicator of oxidative processes in the body, and shows that even in AD individuals the
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39 oxidative processes are enhanced. This is confirmed by the observed decrease of antioxidant
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41 vitamins, α -tocopherol and retinol in blood plasma of AD individuals. Further decrease of
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43 these antioxidants, as well as ascorbic acid was observed in CRC patients (Table 1). This may
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45 suggest that antioxidant defense gradually decreases in the course of disease development.
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47 Other studies show a similar depletion of antioxidant vitamins in cancer patients in
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49 comparison to controls (49). The mechanism of such depletion may involve either insufficient
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51 vitamin intake, or malabsorption in the intestine or accelerated usage during disease
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53 progression. Vitamins E and A may also participate in regulation of cell proliferation, e.g., α -
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55 tocopherol inhibits protein kinase C activity and in effect cell proliferation. Supplementation
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3 of animals with vitamin E prevents chemically induced colon cancer (50). Vitamin A
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5 deficiency is responsible for decreased mucosa production and expansion of proliferation
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7 zones (51). Absorption of vitamins C and E occurs *via* intestinal transporters (52,53).
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9 Moreover, cytochrom P450-catalyzed catabolism of tocopherols may be a decisive factor
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11 responsible for the bioavailability of vitamin E (54).
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15 Since in our study the subjects had similar dietary habits, the differences in antioxidant
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17 vitamin levels might be at least partially genetically determined.
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21 The second line of defense against oxidative stress is DNA repair. 8-OxoGua excision
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23 activity was significantly higher in leukocytes of CRC patients in comparison to healthy
24
25 volunteers (Table III). The increase in 8-oxoGua repair capacity might be due to increased
26
27 transcription from *OGGI* and *APE1* genes, which was observed in AD and CRC individuals
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29 (Table VI). Thus, induction of DNA repair genes seems to occur at an early stage of the
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31 carcinogenic pathway, and may be caused by increased oxidative stress. Expression of *APE1*
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33 and *OGGI* is induced by ROS (26), and we found that oxidative stress is enhanced both in the
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35 CRC and AD group. Our results corroborate the findings of Winnepenninckx and coworkers
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37 (55) that induction of most DNA repair genes occurs very early in the carcinogenic process,
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39 *e.g.*, four years before clinical manifestation of malignant skin melanoma. This may be a part
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41 of a carcinogenic program, which might decrease apoptosis in aberrant cells and/or favor
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43 genomic instability. In yeast and human cells, overexpression of some repair enzymes,
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45 namely methylpurine DNA glycosylase and/or APE1, was associated with frameshift
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47 mutations and microsatellite instability (56).
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54 Increase in 8-oxoGua excision in blood leukocytes of CRC patients was in contrast to
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56 studies of other cancer types, which showed that 8-oxoGua repair capacity was decreased in
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58 lung (23,24) and in head and neck (25) cancer patients. Such a decrease was frequently linked
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60 to the *OGGI* Ser326Cys polymorphism. Pure 326Cys OGG1 variant excises 8-oxoGua and

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3 FapyGua from DNA at lower rates than the wild-type enzyme (28,29). However,
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5 controversial results showing no association between *OGGI* Ser326Cys polymorphism and 8-
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7 oxoGua excision rate **have** also been reported (26). Recent work of Bravard and coworkers
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9 (57) shows that the *OGGI* Cys variant is more sensitive to oxidative inactivation than the Ser
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11 variant, so different degrees of oxidative stress in different studies might partially explain the
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13 controversies in **the** literature.
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17 This study shows a higher frequency of Cys326Cys homozygotes **among** CRC patients
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19 than **among** AD individuals or **the** control group (Table IV). Our results **in contrast to the**
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21 observations of Hansen and coworkers (33), who found **a** higher frequency of Cys allele
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23 among **the** controls than CRC patients. Allelic distribution may depend on genetic
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25 background of **the** local population, specifically if the population is **isolated**, like it was in
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27 Poland after the Second World War till the nineteen nineties. Another study performed on the
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29 Polish population did not find an association between **the** *OGGI* polymorphism and CRC
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31 risk, but **a** higher, although statistically **insignificant**, frequency of the Ser326Cys genotype
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33 was found in CRC patients in comparison to controls ($P=0.07$) (34). Similarly to our results,
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35 Moreno and coworkers (58) showed **an** association between *OGGI* Cys/Cys genotype and
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37 CRC risk in the Spanish population. These inconsistent results regarding the association of **the**
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39 *OGGI* genotype and CRC suggest the influence of other **factors**.
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46 In our study the effect of **the** *OGGI* Ser326Cys polymorphism on 8-oxoGua excision
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48 rate was clearly seen in **the** CRC patient group, in which Cys homozygotes **were found to**
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50 **have a** decreased 8-oxoGua excision rate **in comparison with** Ser homozygotes. Interestingly,
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52 the *OGGI* genotype exerted a limited effect on 8-oxodG level **in leukocytes** and urinary
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54 excretion of 8-oxoGua (Table V). The only difference found was the decreased level of 8-
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56 oxodG in leukocytes of CRC patients with **the** *OGGI* Ser326Cys heterozygous genotype in
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58 comparison to both Cys/Cys and Ser/Ser homozygotes. This is difficult to explain, since
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3 leukocytes of CRC patients with at least one Cys allele exhibited an increased mRNA level of
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5 MTH1 phosphohydrolase (Table VI). MTH1 activity prevents incorporation of 8-oxodGTP to
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7 DNA and decreases the level of 8-oxodG in DNA. Thus, several pathways are engaged in
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9 elimination of 8-oxoGua from DNA, for example nucleotide pool sanitation and base excision
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11 repair (10).
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15 In the group of healthy subjects there was significant positive correlation between
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17 mRNA levels of the main enzymes involved in 8-oxoGua removal (OGG1 and APE1), and
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19 the amount of 8-oxoGua in urine. Therefore, our finding is the first experimental evidence
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21 which suggests that urinary 8-oxoGua measurements may be attributed to DNA repair.
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23 However, there was no such correlation in the groups of adenoma individuals and carcinoma
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25 patients. The main reason for this inconsistency may be aberrant DNA oxidation in cancerous
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27 and precancerous conditions which may overshadow the subtle relationship observed in
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29 healthy subjects.
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34 This study shows that oxidative stress and antioxidant vitamin deficiency are increased
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36 in individuals developing colon adenomas and carcinomas, and may suggest that they
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38 contribute to the development of colon cancer. At the early stage of colon carcinogenesis, a
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40 defense pathway for elimination from DNA of 8-oxoGua and FapyGua is induced. This
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42 induction may, nevertheless, be insufficient to counteract the increased DNA damage.
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48 **Acknowledgments**

49
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51
52 University of Technology, Warsaw, Poland) for help in statistical evaluation of the obtained
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54 results.
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57 **Author disclosure statement**

No competing financial interests exist.

Abbreviations

8-oxodG - 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua – 8-oxo-7,8-dihydroguanine; AD – adenoma; CRC – colorectal cancer; cv – coefficient of variation; FapyGua – 2,6-diamino-4-hydroxy-5-formamidopyrimidine; MPA – metaphosphoric acid; MSSCP - Multitemperature PCR-Single Strand Conformation Polymorphism; ROS – reactive oxygen species; SDS – sodium dodecyl sulfate

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Table I. The concentrations of antioxidants in the plasma of colon cancer patients, individuals with adenomas and healthy volunteers

	Carcinoma patients (C) ^a	Adenoma patients (A) ^a	Healthy volunteers (H) ^a	<i>P</i> ^b
Ascorbic acid (μM)	34.76 (20.01–47.71) n = 89	49.79 (30.13–68.19) n = 77	56.70 (40.68–73.09) n = 99	0.000000 (C vs. H) 0.058 (A vs. H) 0.000032 (C vs. A)
α-Tocopherol (μM)	23.92 (17.87–27.77) n = 86	30.15 (24.95–37.69) n = 71	35.71 (28.71–41.43) n = 101	0.00001 (C vs. H) 0.0083 (A vs. H) 0.000000 (C vs. A)
Retinol (μM)	1.13 (0.85–1.49) n = 85	1.70 (1.39–2.14) n = 71	1.99 (1.69–2.39) n = 101	0.00001 (C vs. H) 0.00015 (A vs. H) 0.00001 (C vs. A)
Uric acid (μM)	277.79 (207.74–351.41) n = 89	325.23 (276.14–393.13) n = 77	312.25 (271.75–381.08) n = 99	0.00011 (C vs. H) 0.70 (A vs. H) 0.00011 (C vs. A)

^a Comparison of antioxidants, expressed as median and interquartile range, between patients groups and healthy volunteers (Mann-Whitney *U* test).

^b All *P* values are after Bonferroni correction.

Table II. The level of oxidized nucleosides in DNA of leukocytes and urine of colon cancer patients, individuals with adenomas and healthy volunteers

	Carcinoma patients (C) ^a	Adenoma patients (A) ^a	Healthy volunteers (H) ^a	<i>P</i> ^b
8-OxodG/10 ⁶ dG in leukocyte DNA	6.31 (4.91–8.27) n = 75	5.89 (4.56–8.57) n = 70	4.41 (3.62–5.71) n = 99	0.000000 (C vs. H) 0.000004 (A vs. H) 0.37 (C vs. A)
8-OxoGua in urine (nmols/mmol creatinine)	10.07 (7.37–15.20) n = 59	7.55 (5.16–11.15) n = 45	7.68 (4.61–10.13) n = 84	0.000075 (C vs. H) 0.51 (A vs. H) 0.0079 (C vs. A)
8-OxodG in urine (nmols/mmol creatinine)	1.74 (1.31–2.99) n = 54	1.70 (1.25–2.95) n = 44	1.38 (1.09–1.73) n = 82	0.00026 (C vs. H) 0.000008 (A vs. H) 0.54 (C vs. A)

^a Comparison of the level of oxidized nucleosides, expressed as median and interquartile range, between patient group and healthy volunteers (Mann-Whitney *U* test).

^b All *P* values are after Bonferroni correction.

Table III. 8-OxoGua repair activity in leukocytes of colon cancer patients and healthy volunteers

8-OxoGua excision activity (pmols/h/mg protein)		<i>P</i>
Carcinoma patients ^a	Healthy volunteers ^a	
40.1 (29.43–59.69) n = 68	22.50 (15.30–29.40) n = 90	0.000000

^a Comparison of 8-OxoGua repair activity, expressed as median and interquartile range, between carcinoma patients and healthy volunteers (Mann-Whitney *U* test).

Table IV. Genotypes and allelic frequencies of the *OGG1* Ser326Cys polymorphism among colon cancer patients, individuals with adenomas and healthy volunteers

OGG1 genotype	Carcinoma patients n = 74 n (%)	Adenoma patients n = 76 n (%)	Healthy volunteers n = 97 n (%), (%) [*]
Cys/Cys	17 (23.0)	1 (1.3)	1 (1), (1) ^a
Ser/Cys	19 (25.7)	29 (38.2)	33 (34), (18) ^b
Ser/Ser	38 (51.3)	46 (60.5)	63 (65), (81) ^c
Allelic frequency			
C	0.52	0.89	0.9
G	0.48	0.11	0.1

^{*} Results from Hardy-Weinberg equilibrium.

^a Homozygous *OGG1* Cys326Cys individuals

^b Heterozygous *OGG1* Ser326Cys individuals

^c Homozygous *OGG1* Ser326Ser individuals

Table V. Comparison of *OGG1* Ser326Cys polymorphism and 8-oxoGua excision capacity of blood leukocytes of CRC patients and healthy volunteers as well as 8-oxodG level in blood leukocytes and urine of CRC patients

OGG1 genotype	Carcinoma patients ^a	Healthy volunteers ^a	<i>P</i>
<i>Repair capacity</i>			
(8-oxoGua pmols/h/mg protein)			
Cys/Cys	24.50 (15.15–42.70) n = 13	–	
Ser/Cys	35.65 (25.50–50.95) n = 14	19.25 (12.4–23.45) n = 32	0.0016
Ser/Ser	42.00 (33.39–86.18) n = 34	24.20 (18.50–31.59) n = 53	0.000000
<i>8-OxodG in blood leukocytes DNA</i>			
(8-oxodG/10 ⁶ dG)			
Cys/Cys	8.02 (5.86–11.80) n = 10	–	
Ser/Cys	5.42 (4.22–8.74) n = 19	4.72 (4.04–6.03) n = 35	0.1
Ser/Ser	6.91 (5.86–8.52) n = 29	4.29 (3.55–5.12) n = 61	0.000001
<i>8-OxoGua in urine</i>			
(nmols/mmol creatinine)			
Cys/Cys	12.83 (7.76–19.14) n = 15	–	
Ser/Cys	10.2 (7.6–13.38) n = 18	8.05 (4–10) n = 36	0.011
Ser/Ser	9.31 (6.35–13.57) n = 27	7.6 (4.95–10.49) n = 47	0.049

^a Comparison of *OGG1* Ser326Cys polymorphism and 8-oxoGua excision capacity as well as 8-oxodG level in carcinoma patients and healthy volunteers, expressed as median and interquartile range (Mann-Whitney *U* test).

Table VI. mRNA level of OGG1 glycosylase, APE1 endonuclease and MTH phosphohydrolase in leukocytes of colon cancer and adenoma patients and healthy volunteers

Genotype	mRNA level (mRNA of gene X/mRNA of 18S rRNA x 10 ⁻⁶) ^a			P
	Carcinoma patients (C)	Adenoma patients (A)	Healthy volunteers (H)	
<i>OGG1</i>				
All	1.28 (0.71–5.58) n = 44	0.99 (0.45–4.91) n = 38	0.19 (0.06–0.54) n = 40	0.000000 (C vs. H) ^b 0.000002 (A vs. H) ^b 0.46 (C vs. A) ^b
Cys/Cys	0.75 (0.26–3.1) n = 8	–	–	–
Ser/Cys	1.26 (0.99–2.87) n = 4	1.01 (0.6–7.16) n = 19	0.48 (0.074–0.63) n = 19	0.021 (C vs. H) ^b (NS) 0.003 (A vs. H) ^b 0.78 (C vs. A) ^b
Ser/Ser	1.99 (0.87–9.59) n = 23	0.97 (0.38–4.77) n = 19	0.1 (0.02–0.23) n = 18	0.000000 (C vs. H) ^b 0.000009 (A vs. H) ^b 0.11 (C vs. A) ^b
<i>APE1</i>				
All	88.25 (42.88–362.51) n = 44	113.83 (48.74–292.37) n = 38	13.87 (2.32–43.04) n = 40	0.000000 (C vs. H) ^b 0.000000 (A vs. H) ^b 0.5 (C vs. A) ^b
Cys/Cys	54.35 (12.36–400.5) n = 8	–	–	–
Ser/Cys	120.6 (61.76–359.7) n = 4	129.54 (72.69–449.52) n = 19	17.67 (3.43–86.3) n = 19	0.021 (C vs. H) ^b (NS) 0.000003 (A vs. H) ^b 0.67 (C vs. A) ^b
Ser/Ser	78.02 (39.56–470.19) n = 23	90.51 (27.46–240.1) n = 19	3.47 (1.07–16.21) n = 18	0.000003 (C vs. H) ^b 0.000005 (A vs. H) ^b 1.00 (C vs. A) ^b
<i>MTH1</i>				
All	86.82 (5.19–728.53) n = 37	–	5.26 (0.33–11.69) n = 19	0.0012
Cys/Cys	96.73 (5.34–295.62) n = 7	–	–	–
Ser/Cys	273.1 (86.82–459.37) n = 2	–	3.7 (0.36–9.88) n = 9	0.036
Ser/Ser	10.84 (1.76–114.65) n = 20	–	8.96 (3.94–26.59) n = 8	0.8

^a Comparison of mRNA levels, expressed as median and interquartile range, between colon carcinoma patients, adenoma patients and healthy volunteers (Mann-Whitney *U* test).

^b *P* values are after Bonferroni correction.

NS, not significant