

RESEARCH ARTICLE

Impact of xanthohumol (a prenylated flavonoid from hops) on DNA stability and other health-related biochemical parameters: Results of human intervention trials

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Scope: Xanthohumol (XN) is a hop flavonoid found in beers and refreshment drinks. Results of *in vitro* and animal studies indicate that it causes beneficial health effects due to DNA protective, anti-inflammatory, antioxidant, and phytoestrogenic properties. Aim of the present study was to find out if XN causes alterations of health-related parameters in humans.

Methods and results: The effects of the flavonoid were investigated in a randomized crossover intervention trial ($n = 22$) in which the participants consumed a XN drink (12 mg XN/P/day). We monitored alterations of the DNA stability in single cell gel electrophoresis assays in lymphocytes and of several health-related biomarkers. A decrease of oxidatively damaged purines and protection toward reactive oxygen species induced DNA damage was found after the consumption of the beverage; also the excretion of 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-oxo-guanosine in urine was reduced. The assumption that the flavonoid causes DNA protection was confirmed in a randomized follow-up study with pure XN ($n = 10$) with a parallel design. Other biochemical parameters reflecting the redox- and hormonal status and lipid- and glucose metabolism were not altered after the intervention.

Conclusion: Taken together, our data indicate that low doses of XN protect humans against oxidative DNA damage.

Keywords:

DNA damage / SCGE assay / 8-oxodG / 8-oxoGuo / xanthohumol



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Abbreviations: ALP, alkaline phosphatase; CRP, C-reactive protein; ENDO III, endonuclease III; FPG, formamidopyrimidine glycosylase; 15F_{2t}-IsoP, 15F_{2t}-Isoprostane; FRAP, ferric reducing ability of plasma; HDL-C, high-density lipoprotein

cholesterol; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; oxLDL, oxidized low-density lipoprotein; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-guanosine; ORAC, oxygen radical absorbance capacity; OC, osteocalcin; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis assay; SC, standard conditions; t-BOOH, t-butylhydroperoxide; XN, xanthohumol

1 Introduction

Xanthohumol (XN, Supporting Information Fig. 1) is a bioactive prenylated flavonoid that was isolated from hop extracts [1]. Gerhäuser and coworkers tested this compound in a battery of *in vitro* assays which was developed for the screening of phytochemicals for cancer protective properties [2, 3]. They found that XN has highly promising properties and postulated that it may inhibit carcinogenesis at the initiation, promotion and progression stage [2]. This assumption is based on earlier findings which indicated that XN inhibits the division of cancer cells [4, 5], possesses anti-inflammatory properties [6, 7] and phytoestrogenic activity [8, 9], acts as a potent antioxidant [1, 10], and protects DNA against chemically induced damage [11].

Evidence for these effects came initially from *in vitro* experiments; it was found that XN is effective in some of these models at very low concentrations. Subsequently, results from animal studies became available which support the assumption of cancer protective properties of the flavonoid. We showed in a recent investigation that XN prevents chemical induction of DNA damage and formation of preneoplastic lesions in livers and colons of rats [12]. Furthermore, it was found that the flavonoid inhibits angiogenesis and oxidative stress [13] and reduces obesity in laboratory animals [14].

Aim of the present study was to find out if beneficial effects can be found also in humans. Therefore, we performed the first placebo controlled intervention trial with XN and investigated if its consumption prevents DNA damage, which plays a key role in the etiology of cancer and other diseases [15]. Additionally, we monitored a variety of health related biomarkers which reflect oxidative stress, the hormonal status, osteogenesis, inflammation, glucose, and lipid metabolism since it was postulated on the basis of results obtained *in vitro* with cultured cells and with rodents that XN may cause beneficial health effects, i.e., it was claimed that the flavonoid may protect against cancer and cardiovascular diseases, prevent postmenopausal discomforts and osteoporosis, and reduce the body weight [16].

Alterations of the DNA stability were measured in single cell gel electrophoresis (SCGE) assays which are based on the determination of DNA migration in an electric field and are frequently used in human intervention studies [17, 18]. The measurements were conducted under standard conditions (SC) (which reflect formation of single and double strand breaks and apurinic sites); with lesion specific enzymes that enable the assessment of endogenously formed oxidatively damaged DNA bases and after ROS (H_2O_2) treatment of the lymphocytes [19, 20]. Additionally, 8-oxo-guanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) levels were measured in urine as additional markers of oxidative damage [21].

Parameters of the redox status which were determined in the participants before and after consumption of the XN drink included isoprostanes ($15F_{2t}$ -IsoP) in urine, oxidized

low-density lipoprotein (oxLDL), the oxygen radical absorbance capacity (ORAC), malondialdehyde levels (MDA) and the ferric reducing ability in plasma (FRAP) [22]. These parameters were monitored as Gerhäuser et al. [3] found that XN is a potent scavenger of peroxy- and hydroxyl radicals under *in vitro* conditions. C-reactive protein (CRP) was monitored as a marker for inflammation [23] and cancer risks [24]. It was shown earlier that the flavonoid reduces inflammation in diabetic rats [13] and inhibits the expression of pro-inflammatory genes in human and rat derived liver cells [25]. Changes of the hormonal status were monitored by determination of the progesterone and 17β -estradiol concentrations in the blood of the participants; as mentioned above, evidence for phytoestrogenic properties was found in several *in vitro* experiments with different cell lines [3, 26]. Alkaline phosphatase (ALP) activity was determined in serum. This enzyme is controlled by estrogen receptors and dephosphorylates proteins involved in cell growth, differentiation, apoptosis, and cell migration. It was shown by Guerreiro and coworkers [27] that XN decreases its expression in MCF-7 cells. Furthermore, changes in the glucose, triglycerol, cholesterol, LDL-cholesterol and HDL-cholesterol concentrations were monitored in plasma as a number of investigations indicate that XN interacts with the glucose and lipid metabolism [14, 28].

To confirm that alterations of individual markers are caused by the flavonoid itself and not by other components of the drink (roast malt extract and malic acid), a small follow-up study was realized in which the participants ($n = 10$) consumed an identical amount of encapsulated pure XN as in the main study.

2 Materials and methods

2.1 Composition of the XN drink and of XN capsules

The XN supplemented drink (commercially not available) was provided by TA-XAN Company (www.xan.com). Around 1000 mL of the beverage contained 12.0 mg of the flavonoid, 1.0 g roast malt extract, 40.0 g sugar, and 0.042 g malic acid. The placebo drink which was prepared contained identical amounts of roasted malt extract, sugar, and malic acid but no XN.

Pure XN (CAS Nr. 6754-58-1) was provided by Hopsteiner (Hallertauer Hopfenveredelung GmbH, Mainburg, Germany). Each XN capsule contained 12.0 mg XN and 422 mg lactose.

The participants consumed in the main study an XN containing nonalcoholic beverage or a placebo. The amount of XN which was consumed by each participant (12 mg/P/day) per day is equal to that used in a previous animal study [12] and is contained in several litres of XN rich beers [1] and in 1 l of the XN drink.

2.2 Recruitment of the participants

Both studies were approved by the Ethical Commission of the Medical University of Vienna (EK-Nr.: 341/2010) and informed consent was obtained from all participants. In total, 22 individuals (11 females and 11 males) participated in the study with the beverage. All of them were healthy nonsmokers who consumed a mixed diet. Two weeks before and during the intervention they did not consume dietary supplements and pharmaceuticals (except contraceptives). Furthermore, they were asked to avoid consumption of beer and foods which contain high levels of flavonoids [29], and to abstain from exhausting physical exercises which may affect the DNA stability and the redox status [30] 1 wk before and during the intervention period and during the wash-out phase. The demographic data of the participants are listed in Table 1.

Participants of the second trial ($n = 10$, five females and five males) fulfilled the same inclusion criteria and had been also enrolled in the first trial.

2.3 Study design and sampling

The design of the randomized intervention trial with the XN drink is depicted in Fig. 1A. The study had a crossover design which has the advantage that seasonal effects which were observed in some SCGE studies can be avoided [31]. The participants consumed daily (within 1 h) either 1 l of the XN supplemented drink or an equivalent amount of an XN free placebo drink in the morning, 3 h before the blood collection. Urine and blood samples were collected at the start, during (4 h, 3 days, 7 days) at the end of the intervention period (14 days) and after a wash-out phase (14 days).

The follow-up study was a randomized intervention trial with parallel design and was not placebo-controlled. The participants consumed capsules that contained pure XN (12.0 mg/P/day) over a period of 14 days. Blood samples were collected before and after the intervention. All participants of the second trial had also been enrolled in the first study.

The drinks and also the XN capsules were consumed by the participants under controlled conditions, i.e., in presence of at least one scientist involved in the realization of the study.

Twenty-four-hour urine samples were collected in the main study at the start and at the end of the trial and were stored deep frozen at -80°C . Blood samples were collected after different time points (start, 4 h, 3 days, 7 days, 14 days) in heparinized tubes (Becton-Dickinson, Plymouth, UK). After centrifugation ($760 \times g$, 10 min, 4°C), the plasma samples were aliquoted and stored at -80°C .

Lymphocytes were isolated with Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) according to the instructions of the manufacturer. Subsequently, suspensions of the cells were aliquoted with Biofreeze (Biochrom AG, Berlin, Germany) in cryo tubes ($1000 \mu\text{l}/\text{tube}$, 1 part cell pellet + 9 parts Biofreeze medium, 10^6 cells/tube) and stored in liquid nitrogen.

Table 1. Demographic data of the participants^{a)}

Parameters ^{b)}	Start			Placebo drink (14 days)			XN drink (14 days)		
	Males ($n = 11$)	Females ($n = 11$)	All ($n = 22$)	Males ($n = 11$)	Females ($n = 11$)	All ($n = 22$)	Males ($n = 11$)	Females ($n = 11$)	All ($n = 22$)
Body weight (kg)	80.8 ± 11.3	66.2 ± 9.9	73.5 ± 12.8	80.6 ± 11.2	66.1 ± 9.7	73.4 ± 12.6	81.3 ± 11.7	66.3 ± 10.1	73.8 ± 13.2
BMI (kg/m^2)	25.0 ± 3.1	24.2 ± 3.6	24.6 ± 3.3	25.3 ± 2.9	23.6 ± 3.9	24.4 ± 3.2	25.2 ± 2.6	25.0 ± 3.1	25.1 ± 2.8
Age (years)	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3	25.0 ± 2.9	27.3 ± 4.3	26.1 ± 4.3
SBP (mmHg)	118.0 ± 12.0	118.1 ± 11.3	118.0 ± 11.6	116.3 ± 11.4	116.1 ± 10.1	116.2 ± 10.7	117.3 ± 12.3	116.5 ± 10.8	116.9 ± 11.5
DBP (mmHg)	77.0 ± 8.2	77.5 ± 7.3	77.3 ± 8.0	77.1 ± 6.7	75.4 ± 6.0	76.2 ± 6.3	77.0 ± 7.7	75.0 ± 6.5	76.2 ± 7.1
Pulse (min.)	80.2 ± 11.8	82.9 ± 13.4	81.5 ± 12.4	79.8 ± 10.1	76.7 ± 11.14	78.2 ± 10.4	82.0 ± 13.4	77.9 ± 8.4	80.0 ± 11.1

^{a)}Numbers indicate means ± SD.

^{b)}BMI, Body Mass Index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

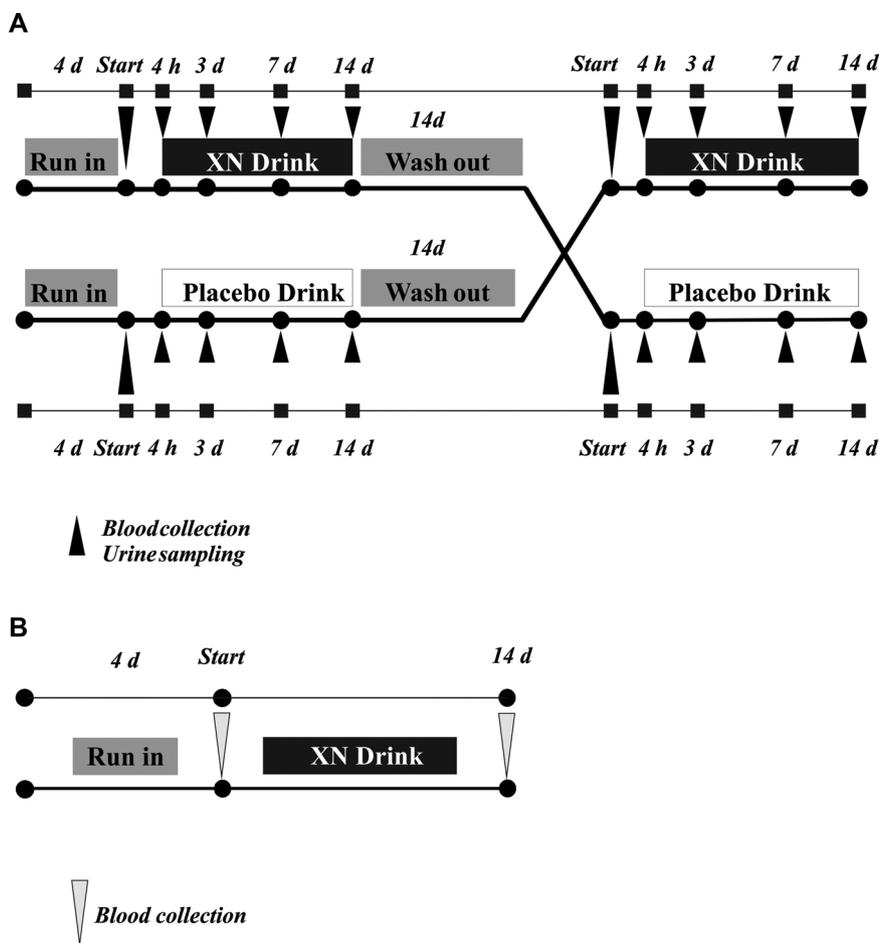


Figure 1. (A) Design of the main intervention trial. Blood and urine samples were collected after different time points (start, 4 h, 3 days, 7 days, 14 days). Twenty-two individuals participated in this trial. (B) Design of the follow-up study with XN capsules ($n = 10$).

Before and during the study, the pulse frequencies, blood pressure, and body weights of the participants were recorded. Additionally, information concerning the impact of the consumption of the beverage and of capsules which contained the flavonoid on the health status was collected with questionnaires.

2.4 Determination of XN in the plasma samples

The concentration of XN in plasma was determined with a Dionex “UltiMate 3000” system (Dionex Corp., Sunnyvale, CA, USA). The column oven was set at 35°C and the UV-Detector at 380 nm. Briefly, after addition of 500 μ l of acetonitrile to 250 μ l of plasma, the samples were centrifuged (5000 \times g, 5 min, 4°C). 80.0 μ l of the sample was injected onto the HPLC column. Separation of XN was carried out using a Hypersil BDS-C18 column (5 μ m, 250 \times 4.6 mm id, Astmoor, England) preceded by a Hypersil BDS-C18 pre-column (5 μ m, 10 \times 4.6 mm id) at a flow rate of 1 ml/min. The mobile phase consisted of a continuous linear gradient, mixed from 10 mM ammonium acetate/acetic acid buffer, pH 5.0 (mobile phase A), and ACN (mobile phase B). The gradient ranged

between 20 % B (0 min) and 80 % at 20 min, stayed constant at 80 % until 25 min, and finally decreased linearly to 20 % at 27 min. The columns were allowed to re-equilibrate for 9 min between runs. Linear calibration curves were established from the peak areas of XN to the external standard by spiking drug-free human serum with standard solutions of XN (final concentrations ranging from 0.005 to 10.0 μ g/ml). The limit of quantification for XN was 0.005 μ g/ml with coefficients of accuracy and precision < 9%.

2.5 Determination of XN concentrations in the XN drink

The concentration of XN in the beverage was determined at the start and after 1, 2, 3, 4, and 6 weeks with a Dionex “UltiMate 3000” system (Dionex Corp., Sunnyvale, CA, USA) as described above using an identical column, column temperature and mobile phase A and B. All bottles of the XN drink were stored at 4°C and immediately opened before analysis. The drinks were filtered through a 0.45 μ m filter (Millipore, Vienna, Austria) and 80.0 μ l of the filtrate injected into the HPLC column. Linear calibration curve for XN was

established from the peak area of XN to the external standards by spiking flavonoid free beverage provided by Hopsteiner (Hallertauer Hopfenveredelung GmbH, Mainburg, Germany) with standard solution of XN (Sigma Aldrich, Munich, Germany) (final concentrations ranging from 0.005 to 20.0 µg/ml). The limit of quantification for XN was 0.005 µg/ml with coefficients of accuracy and precision < 8.2 %. The beverage which was stored at 4°C was quite stable over 6 weeks with a slight decrease of the XN content to 92.14%. The XN concentration in the drink was 13.37 ± 0.45 mg/l.

2.6 DNA damage

2.6.1 SCGE assays with human lymphocytes

The experiments were carried out according to the guidelines for SCGE experiments [19]. Frozen cells were thawed and centrifuged (8 min, $110 \times g$) twice in phosphate-buffered saline (PBS, pH 7.4). The vitality of the cells was determined in each experiment with the trypan blue (0.4%) dye exclusion technique [32] with an improved Neubauer hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). DNA damage was only analyzed in cells from samples in which the vitality was $\geq 80\%$ as acute cytotoxic effects may cause false positive results [33]. The cells were mixed with 0.5% low melting point agarose and transferred to agarose coated slides (1.0% normal melting point agarose). In comet assays, electrophoresis was carried out under alkaline conditions (30 min, 300 mA, 1.0 V/cm, at 4 °C, pH > 13) after lysis (pH 10.0). Subsequently, the slides were neutralized and dried slides were stained with ethidium bromide (20.0 µg/ml, Sigma-Aldrich, Steinheim, Germany).

Additionally, experiments were conducted in which intact cells were treated with H₂O₂. After exposure to 50 µM (on ice) for 5 min, slides were rinsed with PBS and further processed as described above. Control slides were exposed in these experiments to PBS (Ca and Mg free, PAA Laboratories GmbH, Pasching, Austria).

To determine formation of oxidatively damaged DNA bases, the nuclei were exposed after lysis to formamidopyrimidine glycosylase (FPG, Sigma-Aldrich, Steinheim, Germany) which detects oxidatively damaged purines, or to endonuclease III (ENDO III, Sigma-Aldrich, Steinheim, Germany) which enables to assess the formation of oxidized pyrimidines. To establish the optimal amounts of the enzymes, calibration experiments were carried out with blood cells from three donors according to the protocol of Collins et al. [34] before the main experiments.

After lysis, the slides were washed twice with enzyme reaction buffer (pH 8.0) for 8 min. Subsequently, the nuclei were treated with 50 µl of the enzyme solutions or with the enzyme buffers. The incubation time for FPG was 30 min and for ENDO III 45 min at 37°C, respectively. After the treatment, electrophoresis was carried out under SC (30 min, 300 mA, 1.0 V/cm, at 4°C, pH > 13) for details see Collins

and Dusinska [20]. In all experiments, parallel measurements were included, in which the nuclei were treated with the enzyme buffers only. After lysis and electrophoresis, slides were evaluated as described above.

From each participant, three slides were prepared per experimental point and from each slide, 50 cells were evaluated. Slides were examined under a fluorescence microscope (Nikon EFD-3, Tokyo, Japan) using 25-fold magnification. DNA migration was determined with a computer aided comet assay image analysis system (Comet Assay IV, Perceptive Instruments, UK).

In all SCGE experiments, internal control measurements were included with aliquots of deep frozen lymphocytes from a healthy donor who did not participate in the intervention trial. DNA migration was monitored in these cells in each experiment under SC and after treatment of the nuclei with restriction enzymes or with enzyme buffers and no anomalous enzyme behavior was observed (data not shown).

Supporting Information Fig. 4 shows the result of a further control experiment with FPG which was conducted once immediately before the experiments with a human derived buccal cell line (TR-146) with a chemical (RO 19–8022, 1 µM, Chiron As, Trondheim, Norway), which causes formation of oxoguanine; the results confirm that the enzyme which was used was active.

2.6.2 Measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-guanosine (8-oxoGuo) in urine

8-OxodG and 8-oxoGuo were determined in urine. Briefly, a chromatographic separation was conducted on an Acquity UPLC system (Waters, MA, USA), using an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1×100 mm) and a mobile phase of ammonium acetate (2.5 mM/l, pH 5) and ACN. MS/MS detection was conducted with API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) using gel electrospray ionization. As internal standards ¹⁵N5-8-oxoGuo and ¹⁵N5-8-oxodG were applied [35].

2.7 Biochemical parameters which reflect the redox status

2.7.1 Ferric reducing ability of plasma

The FRAP assay is based on the measurement of the reduction of a ferric tripyridyltriazine complex (Fe³⁺) to a ferrous complex (Fe²⁺) at pH 3.6. Changes in absorbance are directly related to the reducing power of antioxidants present in the samples. The measurements were conducted as described by Benzie and Strain [36].

2.7.2 15F_{2t}-Isoprostanes

15F_{2t}-IsoP was determined in urine with a commercially available ELISA kit (Oxford, MI, USA) as previously described [37, 38] according to the instructions of the manufacturer. Urinary creatinine was determined with the Jaffé reaction [39].

2.7.3 Malondialdehyde

MDA levels were determined in plasma according to the method of Ramel et al. [40]. The samples were neutralized after heating (60 min, 100°C) with methanol/NaOH, centrifuged (3 min, 3000 rpm); subsequently MDA was measured with HPLC (excitation: $\lambda = 532$ nm, emission: $\lambda = 563$ nm, LaChrom Merck Hitachi Chromatography system, Japan). Each sample was measured in duplicate.

2.7.4 Oxygen radical absorbance capacity

The ORAC assay was conducted with plasma samples. It is based on the inhibition of peroxyradical induced oxidation which is triggered by thermal decomposition of the azo-compound (2,2'-azo-bis 2-amidino-propane dihydrochloride). The measurements were performed according to the protocol of Cao et al. [41].

2.7.5 Oxidized low-density lipoprotein

Plasma oxLDL concentrations were measured in plasma with a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden) according to the instructions of the manufacturer. Absorbance of the samples and of the standards was determined with a fluorimeter (BMG Lab Technologies, Offenburg, Germany).

2.8 Parameters related to energy metabolism

2.8.1 Glucose

Glucose was measured enzymatically according to the protocol of Keilin and Hartree [42]. This method is based on the oxidation of glucose by purified glucose oxidase.

2.8.2 Cholesterol and lipoproteins

Cholesterol and triglycerol were determined with an enzymatic colorimetric method according to Bucolo and David [43] with a chemical analyzer (AVIDA 2400, Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany). HDL-C was measured with a turbidimetric immunoassay

[44]. LDL-C was estimated as described by Friedewald et al. [45].

2.9 Parameters associated with inflammation

2.9.1 C-reactive protein

CRP was monitored in plasma with a turbidimetric immunoassay according to Sisman et al. [46]. The measurements were conducted with an automated analyzer Olympus OSR6299 (Melville, NY, USA).

2.9.2 Urea

Urea was determined enzymatically by use of urease described by Wilcox et al. [47]. The measurement is based on a colorimetric method. The measurements were conducted with an automated analyzer (AVIDA 2400, Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany). All reagents were purchased from Siemens Healthcare Diagnostics.

2.9 Hormonal status and hormone related parameters

2.9.1 Estradiol and progesterone

Serum 17 β -estradiol and progesterone levels were measured with the ADVIA Centaur automated system (Siemens Healthcare Diagnostics GmbH, Schwalbach am Taunus, Germany) with chemiluminescence immunoassays according to the instructions of the manufacturers [48].

2.9.2 ALP and osteocalcin

Serum ALP was determined with enzyme kinetic measurements according to Hausamen et al. [49]. Osteocalcin (OC) was monitored with commercial electrochemiluminescence immunoassays on an Elecsys 2010 automated analyzer (Roche diagnostics, Mannheim, Germany) measuring N-Mid-fragment and intact OC in serum.

2.10 Statistical analyses

2.10.1 Single cell gel electrophoresis assays

The means and SD of % DNA in the comet tails of the different groups in the main study were calculated on the basis of the results of the individual measurements. The extent of DNA migration attributable to FPG and ENDO III sensitive sites was calculated by subtracting the corresponding enzyme

buffer values that were determined in all experiments. Comparisons of groups were done by ANOVA based on the means of results obtained with three slides from each participant. For each experimental condition, three slides were made in parallel and 50 cells were evaluated per slide. *Post hoc* comparisons between groups (placebo versus XN) were assessed by Dunnett's test. In the second intervention trial with capsules containing pure XN, the statistical analyses were conducted with Student's *t*-tests for comparisons before and after supplementation. *P*-values ≤ 0.05 were considered as significant.

2.10.2 Biochemical parameters

All biochemical parameters were measured either in duplicate or in triplicate for each participant at different time points. Statistical significance was analyzed by Student's *t*-test. For all comparisons, *p*-values ≤ 0.05 were considered as significant. Statistical analyses were performed using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Compliance and impact of consumption of the beverage on the health status

The consumption of the beverage did not cause health problems according to the information collected with questionnaires. All participants consumed the beverage under controlled conditions (i.e., in presence of one of the scientists involved in the trial) and finished the study. Body weight, blood pressure, and pulse were measured at the beginning, during and at the end of the intervention trial. Values which were recorded at the start and at the end of the intervention with the XN drink and with the placebo are listed in Table 1. It can be seen, that no significant changes of the different parameters were found.

3.2 Determination of XN concentrations in the XN drink and in plasma

The stability of flavonoid was monitored in the XN drink over a period of 6 wk, the results are shown in Supporting Information Fig. 2. It can be seen that the XN concentration decreased only slightly during the storage (i.e., to 92.14 % after 6 weeks).

XN was detected in plasma up to 4 h after consumption of the beverage (average concentration in the intervention group 6.13 ± 5.4 ng/ml), at latter time points the levels dropped below the detection limit. This pattern was also seen after repeated consumption of the drink. It is notable that this value is only a crude assessment as it is close to the detection limit and XN could be not detected in 12 samples. Also in an intervention trial from Legette et al. [50] with XN capsules

extreme low levels of free XN were detected in the plasma of volunteers. A typical chromatogram that was obtained with a plasma sample is depicted in Supporting Information Fig. 3.

3.3 Alterations of the DNA stability

The results of SCGE assays with lymphocytes are summarized in Fig. 2A–D. It can be seen that no significant alteration of comet formation was found when the cells were analyzed under SC which reflect single and double strand breaks and apurinic sites. However, in experiments with FPG, significant DNA-protection was seen after consumption of the XN drink. The effects increased with the duration of the intervention period. After 2 wk of continuous consumption of the XN drink, the extent of DNA migration was significantly reduced by 33% ($p < 0.001$, Fig. 2C). On the contrary, no alterations of DNA migration were found after treatment of the nuclei with the second lesion specific enzyme ENDO III (Fig. 2D).

The findings that were obtained after exposure of the cells to ROS are depicted in Fig. 2B. Exposure to H_2O_2 led to a strong increase of DNA damage over the background level. After consumption of the XN drink, a pronounced decrease of the extent of H_2O_2 induced comet formation was observed, i.e., significant reduction of the % DNA in the tails by 53% ($p < 0.05$).

The protective effects (decreased formation of FPG sensitive sites which reflect oxidatively damaged purines and decreased sensitivity towards ROS induced DNA damage) disappeared at the end of the wash-out period. Note that the bars concerning the wash-out phase that are shown in Fig. 2 show results obtained with 11 subjects that consumed XN in the first phase.

Fig. 2A–D show also the results which were obtained with the placebo drink; it can be seen that no significant alterations were found in the different experimental series.

With pure XN which was consumed by the participants in capsules in a follow-up intervention trial, similar effects were found as with the XN drink. Significant protection of DNA damage was seen after 14 days consumption of the flavonoid after treatment of the nuclei with FPG (reduction of comet formation by 39%, $p < 0.001$) and in experiments with H_2O_2 (reduction of comet formation by 28%, $p < 0.05$).

To find out if consumption of the XN drink has also an impact on the excretion of oxidized bases, additional measurements of 8-oxodG and 8-oxoGuo were conducted with urine samples. It can be seen in Fig. 3A and B that a significant reduction of the concentrations of both parameters was observed at the end of the study. The level of 8-oxodG decreased from 21.8 ± 13.7 to 12.2 ± 5.9 nM. Also the levels of 8-oxoGuo declined after consumption of the XN drink (from 27.5 ± 18.0 to 16.2 ± 7.0 nM) after 14 days. These values are means \pm SD of results obtained with samples from 22 participants (*t*-test, $p < 0.05$). The wash-out results are based on measurements that are conducted with samples from 11 participants.

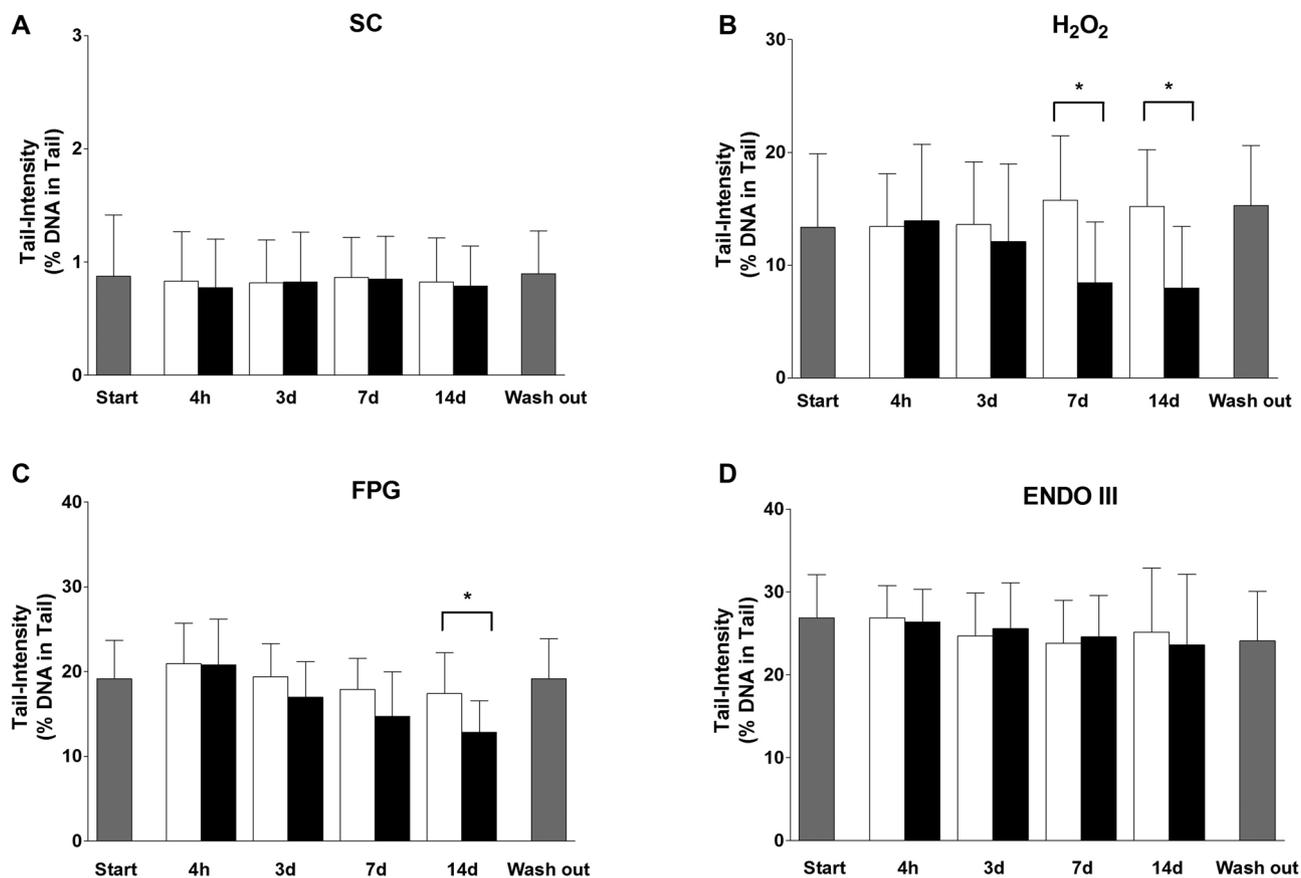


Figure 2. Impact of consumption of the XN supplemented beverage (black bars) and of an XN free (placebo) drink (white bars) on DNA damage in peripheral lymphocytes. The participants ($n = 22$) consumed daily 1 l of the beverages over a period of 14 days. Blood samples were collected before, during, and after the intervention (for details see Fig. 1). DNA migration was measured in SCGE assays under standard conditions (SC) (A) and after exposure of intact cells to ROS ($50.0 \mu\text{M H}_2\text{O}_2$) on ice for 5 min (B), after treatment of the nuclei with FPG (C) and ENDO III (D). For each experimental point, three slides were made in parallel from each participant and 50 cells were analyzed from each slide. Bars show means \pm SD of results which were obtained with 22 individuals after different time intervals; bars which concern the wash-out phase are means \pm SD of results obtained with 11 individuals who consumed the XN drink in the first phase. In the case of the results obtained with lesion specific enzymes, the corresponding buffer values were subtracted. Asterisks indicate statistical significance ($p \leq 0.05$, ANOVA).

3.4 Health related biochemical parameters

The results of measurements of the different biochemical parameters are summarized in Tables 3 and 4. It can be seen that none of them was significantly altered after consumption of the flavonoid.

4 Discussion

This article describes the results of the first placebo controlled human intervention trial in which the impact of consumption of the flavonoid XN on health related biomarkers was monitored. All earlier investigations with humans concerned the pharmacokinetics and tissue distribution of the flavonoid [50–52]. The daily dose that was consumed by the participants in the present study is in the same order of magnitude as the

levels which are reached after consumption of XN rich beers and of nonalcoholic refreshment drinks. Taken together, we found clear evidence for pronounced protection of the genetic material against oxidative DNA damage. However, other health related biomarkers that reflect changes of the energy and lipid metabolism, inflammation and the hormonal status were not significantly altered at the end of the intervention.

The results of the chemical analyses show that the plasma levels of XN decrease shortly after oral intake of the flavonoid; this observation is in agreement with earlier human studies [50], and it is notable that a number of metabolites has been identified [50, 52, 53]. If and to which extent they contribute to the biological effects of XN in human is not known at present. As described in the results section (Fig. 2), we observed pronounced reduction of FPG sensitive lesions after the intervention by 33%. Furthermore, we detected also a clear decrease of ROS (H_2O_2) induced comet formation

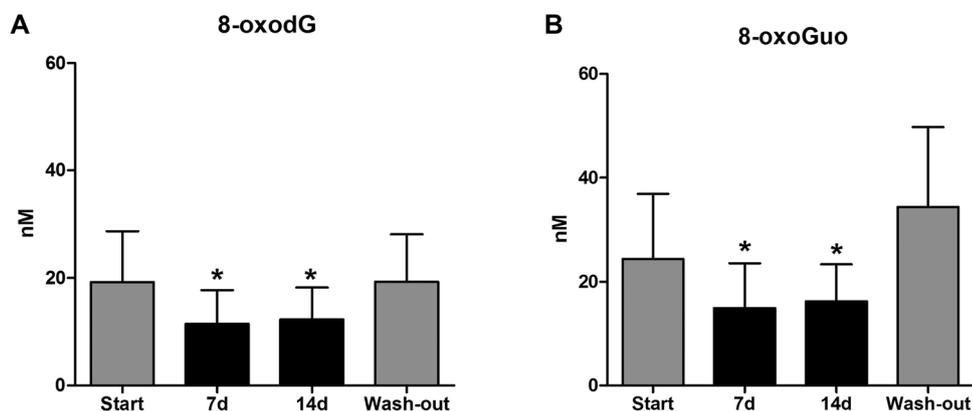


Figure 3. Impact of consumption of the XN drink on formation of urinary 8-oxodG, 8-oxoGuo. Bars show means \pm SD of results obtained with urine samples of 22 participants at the start and after 7 and 14 days; Bars for wash-out period show the results obtained with 11 individuals (for details see Section 2). Asterisks indicate significance (t -test, $p < 0.05$).

(by 53%); this result supports the assumption that XN prevents formation of oxidized purines as the peroxide causes preferentially oxidation of these bases. The protective effects increased with the duration of the intervention time. Other endpoints which are indicative for DNA damage, i.e. comet formation under SC reflecting single and double strand breaks and DNA migration attributable to ENDO III sensitive sites (which are caused by oxidatively damaged pyrimidines) were not affected by XN. No effects were seen in the placebo group at any time point. The findings that were obtained in the follow-up study with XN capsules show that the protective effects are caused by the flavonoid. The results of the FPG experiments which are indicative for prevention of formation of oxidized purine bases are supported by findings that were obtained in HPLC measurements with urine samples showing that intake of XN leads to substantial reduction of the excretion of 8-oxoGuo and 8-oxodG.

It is notable, that DNA protective effects of XN were reported earlier by Plazar et al. [4, 54] who performed *in vitro* experiments with liver slices and additionally with a human derived liver cell line (HepG2). They found reduced comet formation with *t*-butyl-hydroperoxide (*t*-BOOH) when they added low doses of XN (3.5–35.4 ng/ml) to the culture which were close to the serum levels which we detected in the participants (6.13 ng/ml). These findings are of interest in regard to the present study as *t*-BOOH causes oxidative DNA damage [55]. The same group published additional findings that indicate that XN protects also against DNA damage caused by genotoxic carcinogens such as heterocyclic aromatic amines and polycyclic aromatic hydrocarbons [4, 5, 56]; also in these experiments, protective effects were seen with low concentrations.

Two modes of action may account for the prevention of oxidative DNA damage by XN, namely direct scavenging of radicals or (indirect) induction of ROS protective enzymes. Theoretically, it is also possible that the flavonoid may cause protective effects *via* induction of DNA repair processes; however, this mode of action can be excluded as the lymphocytes were analyzed immediately after short exposure (5 min) to hydrogen peroxide on ice, i.e., under experimental conditions where no repair takes place. Several *in vitro* studies showed

that XN prevents formation of oxLDL, reduces the ORAC, and inactivates NO as well as O₂ radicals [2, 3]. These and other *in vitro* experiments were conducted with models that do not reflect the induction of antioxidant enzymes and were carried out with concentrations that exceed the serum levels of the flavonoid which we detected in the blood of the participants after consumption of the XN drink by two to three orders of magnitude. The findings that were obtained in these studies indicate that XN is (like many other flavonoids), able to inactivate reactive ROS *via* scavenging. However, the doses that were required to prevent induction of oxidative DNA damage by *t*-BOOH *in vitro* were much lower (see above) and protective effects were only seen when the indicator cells were exposed for several hours to XN before treatment with the genotoxin, but not when a simultaneous treatment protocol was used [4]. Since antioxidant enzymes are represented in inducible form in the indicator cells (HepG2) which were used in this study [57], it is likely, that the DNA protective effects are due to their upregulation. In this context, it is notable that induction of ROS protective enzymes (superoxide dismutase, glutathione *S*-transferase, and glutathione peroxidase) and of glutathione (which is a potent ROS scavenger) was found in *in vivo* experiments with rats and also with tupaia after oral administration of XN [10, 58]. Recently, a paper was published by Yao and co-workers [59], who found that XN activates in neuronal cells Nrf2 which controls the transcription of a variety of phase II and antioxidant enzymes; this observation could explain the pronounced ROS protective properties of the flavonoid.

As mentioned in the results section, ENDO III-sensitive sites were not markedly decreased after the intervention trial in contrast to FPG-sensitive sites. This difference may be due to the fact that the induction of antioxidant enzymes (see above) may lead to more pronounced protection of purines. In this context, it is notable that oxidized purines are found in healthy humans in substantially larger amounts than pyrimidines [60]. Another possible explanation may be, that the FPG does not only detect damage of oxidized bases but other modes of action; it is well known that the enzyme recognizes a relative broad spectrum of lesions including those caused by alkylating bases [61, 62].

Table 2. Impact of consumption of XN capsules ($n = 10$) on ROS sensitivity and on formation of FPG sensitive sites in lymphocytes^{a)}

SCGE assay (% DNA in tail)	Start	14 days
H ₂ O ₂	21.6 ± 1.1	15.6 ± 1.9 *
FPG ^{b)}	13.0 ± 4.0	7.9 ± 2.7 *

^{a)}The participants ($n = 10$, five females and five males) consumed one XN capsule (12 mg) per day over a period of 14 days (for details see Section 2). Values indicate means ± SD of results obtained from three slides from each participant. Asterisks (*) indicate statistical significance ($p \leq 0.05$, t -test).

^{b)}Values indicate the results obtained after subtraction of the corresponding enzyme buffer values from the values obtained after treatment of the nuclei with the lesion specific enzyme.

The SCGE assay with lesion specific enzymes and/or ROS (H₂O₂) treatment has been used in a number of earlier human intervention trials [17, 31, 63, 64]. Protective effects were seen with several foods and beverages and also with selected vitamins and other bioactive food constituents. Comparisons of the present results with findings of earlier human intervention trials show that XN is highly effective in regard to prevention of oxidative DNA damage in lymphocytes, i.e., significant protection was detected already with an extremely low dose. However, ROS cause oxidative damage of tissues which are in close vicinity of sites where they have been generated and further investigations have to be realized concerning the organ-specific antioxidant properties of XN.

As mentioned above, it was postulated that XN may cause beneficial effects in humans due to its phytoestrogenic activities and that it may reduce the risks for cardiovascular diseases and cause weight loss due to reduced uptake of glucose from the GI-tract *via* changes of the lipid metabolism and prevention of inflammation [16]. It can be seen in Table 2 that we found no evidence for such effects in the present experiments. The main reasons for the discrepancy of our results with findings of older investigations are most likely differences in the dosage. The dose that was used in the present human study was 0.17 mg/kg b.w./day which compares with 1.05 mg/kg b.w./day in mice which was used by Nozawa and coworkers, who found reduction of plasma glucose and hepatic triglycerides in KK-A^y mice [65]. The daily amount which was required to cause a significant reduction of the plasma glucose levels in rats was 16.9 mg/kg b.w./day while lower amounts (5.64 and 1.86 mg/kg b.w./day) were not effective [66].

Also in *in vitro* experiments, high concentrations were required to cause protective effects. For example a concentration of 5 µg/ml was found to cause activation of the farnesoid X receptor (which plays a key role in lipid and glucose metabolism) [65]. This level is approximately 300-fold higher than the peak plasma concentrations that were detected in the participants in the present study. In regard to alterations of the hormonal status, which were attributed to the

Table 3. Impact of consumption of the XN drink on biochemical parameters reflecting the redox status, glucose and lipid metabolisms, and inflammation [6]

Parameters ^{b)}	Units	Placebo drink							XN drink						
		Start	4 hours	3 days	7 days	14 days	Wash out	4 hours	3 days	7 days	14 days				
Redox status															
FRAP	µM/l	335.9 ± 109.4	395.8 ± 126.2	367.8 ± 111.7	376.3 ± 69.3	366.6 ± 150	372.0 ± 124.0	361.6 ± 88.63	337.6 ± 72.3	353.8 ± 109.6	351.8 ± 118.8				
MDA	µM/l	1.4 ± 0.7	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.5	1.5 ± 0.5	1.5 ± 0.4	1.3 ± 0.5	1.4 ± 0.5	1.3 ± 0.5				
ORAC	mM TE/l	3.5 ± 1.1	3.9 ± 0.4	3.8 ± 0.4	3.4 ± 0.6	3.7 ± 0.9	3.1 ± 0.5	4.0 ± 0.7	3.8 ± 0.3	3.5 ± 0.5	3.6 ± 0.4				
OxLDL	U/l	54.3 ± 24.3	43.4 ± 13.9	59.2 ± 27.4	52.2 ± 35.7	38.1 ± 14.1	52.5 ± 33.1	45.2 ± 18.0	55.6 ± 17.6	45.3 ± 17.9	50.8 ± 17.7				
Glucose & lipid status															
Glucose	mg/dl	84.3 ± 23.0	93.0 ± 17.0	90.0 ± 15.2	86.3 ± 13.2	80.8 ± 19.8	86.8 ± 20.7	91.8 ± 16.2	95.3 ± 13.2	84.8 ± 13.8	85.0 ± 12.4				
Triglycerol	mg/dl	91.3 ± 62.0	122.4 ± 50.9	109.3 ± 63.1	100.9 ± 52.5	111.6 ± 76.5	100.2 ± 51.7	120.1 ± 49.3	95.9 ± 38.1	102.1 ± 53.2	124.4 ± 50.5				
LDL-C	mg/dl	89.0 ± 32.18	87.6 ± 22.1	90.0 ± 19.9	88.4 ± 19.2	82.7 ± 25.1	87.0 ± 19.7	89.6 ± 25.2	90.9 ± 23.0	89.1 ± 22.1	85.0 ± 27.8				
HDL-C	mg/dl	54.0 ± 19.6	58.1 ± 12.2	56.9 ± 14.2	56.5 ± 13.2	52.1 ± 18.1	53.7 ± 17.1	54.0 ± 13.7	54.2 ± 13.3	56.2 ± 14.1	57.2 ± 14.6				
Cholesterol	mg/dl	161.4 ± 50.4	170.3 ± 26.9	168.8 ± 30.8	165.2 ± 28.4	157.3 ± 46.39	160.9 ± 31.8	166.5 ± 31.1	165.0 ± 26.7	167.4 ± 29.8	171.6 ± 30.0				
Inflammation status															
Urea	mg/dl	26.0 ± 8.5	25.5 ± 6.8	24.6 ± 6.0	25.9 ± 4.3	21.4 ± 6.4	25.6 ± 8.3	25.4 ± 6.2	23.0 ± 5.8	25.3 ± 5.7	23.4 ± 8.3				
CRP	mg/dl	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	0.2 ± 0.4	0.2 ± 0.4	0.3 ± 0.7	0.1 ± 0.2	0.1 ± 0.2				

^{a)}Values represent means ± SD. All measurements were carried out with plasma. Laboratory diagnostic reference ranges are according to Neumeister et al., [79]: glucose (70–100 mg/dl) triglycerol (< 150 mg/dl); LDL-C (70–100 mg/dl); HDL-C (40–60 mg/dl); cholesterol (< 200 mg/dl); urea (10–50 mg/dl); CRP (< 0.5 mg/dl).

^{b)}CRP, C-reactive protein; FRAP, ferric reducing ability of plasma; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; oxLDL, oxidized low density lipoprotein; TE, trolox equivalent.

Table 4. Impact of consumption of the XN drink on biochemical parameters reflecting the hormonal status and lipid peroxidation II^{a)}

Parameters	Samples	Units	Start			XN drink (14 days)		
			Males (n = 11)	Females (n = 11)	All (n = 22)	Males (n = 11)	Females (n = 11)	All (n = 22)
Hormonal status & hormone related parameters	serum							
Alkaline phosphatase		U/l	60.7 ± 11.5	85.8 ± 14.5	73.9 ± 18.2	64.8 ± 14.4	83.0 ± 13.8	73.9 ± 16.6
Osteocalcin		ng/ml	20.3 ± 5.9	26.5 ± 8.2	23.5 ± 7.7	20.9 ± 6.3	24.1 ± 5.4	22.5 ± 6.0
Progesterone		ng/ml	0.7 ± 0.1	1.3 ± 1.0	1.0 ± 0.7	0.7 ± 0.1	1.1 ± 1.4	0.9 ± 1.0
17β-Estradiol		pg/ml	41.9 ± 13.7	35.6 ± 40.5	38.7 ± 29.6	33.2 ± 15.6	55.1 ± 39.5	44.1 ± 31.4
Lipid peroxidation 15-F _{2t} -IsoP ^{b)}	urine	ng/mg creatinine	1.2 ± 0.5	1.9 ± 0.8	1.5 ± 0.7	1.5 ± 0.6	2.1 ± 0.9	1.8 ± 0.8

^{a)} Values represent means ± SD. All measurements were carried out in plasma or urine and were measured in triplicate. Laboratory diagnostic reference ranges are: alkaline phosphatase (males: 40–130 U/l, females: 35–105 U/l); osteocalcin (males: 11–70 ng/ml, females: 11–43 ng/ml); progesterone (males: 0.2–1.4 ng/ml, females: 0.2–27 ng/ml); 17β-estradiol (males: 14–55 pg/ml, females: 30–150 pg/ml) [79].

^{b)} 15-F_{2t}-IsoP, 15-F_{2t} isoprostane.

phytoestrogenic properties of XN and other structurally related hop flavonoids, the situation is similar, i.e. the doses that were required to cause effects in human derived breast cancer cells were higher than the levels which were found in the serum of the participants [3]. As shown in Table 3, no alterations of the progesterone and 17β-estradiol concentrations were found at the end of the intervention phase. Since estrogens have an impact on bone formation it was hypothesized that prenylated flavonoids may prevent osteoporosis; furthermore, also other mechanisms were discussed which may affect this process (for review see Liu et al. [16]). Indeed, it was found that XN affects osteoblast differentiation and induction of ALP which is a marker for osteogenesis in several studies [3, 67, 68]. All these effects were detected under *in vitro* conditions with XN doses which are at least 15-fold higher than the serum levels of the participants in our study. This discrepancy between the plasma concentrations and the high levels used in *in vitro* experiments provides a plausible explanation for the lack of an effect in the present investigation. In regard to the impact of XN on breast cancer risks which is increased by estrogens, it is notable that Bolca et al. [51] published results of a human study which concerned the distribution of prenylflavonoids in breast tissue; the authors come to the conclusion, that it is unlikely that consumption of low levels (6.12 mg/P/d) causes responses which are mediated *via* estrogen receptors. In a trial with postmenopausal women, a trend was observed for alleviation of postmenopausal discomforts with a hop extract (which contained various prenylated flavonoids), but the effects were not dose dependent and alterations of the hormonal status were not monitored [9].

The most interesting result of the present study is the observation of protection of the genetic material against

oxidative damage (see Fig. 2). Although no data from human studies are available which concern direct associations between oxidation of DNA bases and cancer risks, results which were obtained with genetically modified animals show that mutations in genes encoding for enzymes which are involved in the repair of oxidatively induced DNA damage lead to tumors in different organs [69]. However, a recent comprehensive analysis of human studies shows that DNA repair polymorphisms *per se* play a less important role as compared to gene/environment and gene/lifestyle interactions [70]. The present investigation was conducted with healthy participants and more pronounced effects and health benefits can be expected in individuals with chronic inflammation, adipositas and metabolic diseases such as diabetes type II since these disorders are characterized by increased levels of oxidative damage [71–74]. Furthermore, it is also notable, that higher cancer rates were seen in humans with polymorphisms in such genes [15, 75]. Another relevant area, apart from cancer, is neurological disorders since several recent findings indicate that oxidative DNA damage may play a role in the etiology of neurodegenerative diseases such as Alzheimer, Parkinson, and amyotrophic lateral sclerosis [76]. The fact that XN was found to be extremely active in regard to protection of oxidative DNA damage in humans makes it a promising candidate for the chemoprevention of cancer and neurodegenerative diseases and further investigations in this direction should be realized.

F.F., H. Al-S., A.N., and C.P. conducted and evaluated the SCGE experiments; T.S., R.M., and K.H.W. measured different biochemical parameters; W.J. determined concentrations of XN in plasma, stability of XN-drink and produced XN capsules; M.B. isolated pure XN; measurements of 8-oxodG and

8-oxoGuo were carried out by H.P. and T.H.; 15F2t-IsoP in urine was measured by R.B. and V.R.; M.M. and M.K. performed the statistical analyses; S.K. planned the study and wrote the manuscript.

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The authors have declared no conflict of interest.

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