RESEARCH ARTICLE

Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules

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Scope: Coffee is among the most frequently consumed beverages. Its consumption is inversely associated to the incidence of diseases related to reactive oxygen species; the phenomenon may be due to its antioxidant properties. Our primary objective was to investigate the impact of consumption of a coffee containing high levels of chlorogenic acids on the oxidation of proteins, DNA and membrane lipids; additionally, other redox biomarkers were monitored in an intervention trial.

Methods and results: The treatment group ($n = 36$) consumed instant coffee co-extracted from green and roasted beans, whereas the control consumed water (800 mL/P/day, 5 days). A global statistical analysis of four main biomarkers selected as primary outcomes showed that the overall changes are significant. 8-Isoprostaglandin F2α in urine declined by 15.3%, 3-nitrotyrosine was decreased by 16.1%, DNA migration due to oxidized purines and pyrimidines was (not significantly) reduced in lymphocytes by 12.5 and 14.1%. Other markers such as the total antioxidant capacity were moderately increased; *e.g.* LDL and malondialdehyde were shifted towards a non-significant reduction.

Conclusion: The oxidation of DNA, lipids and proteins associated with the incidence of various diseases and the protection against their oxidative damage may be indicative for beneficial health effects of coffee.

Keywords:
Antioxidants / Chlorogenic acids / Instant coffee / Intervention trial

1 Introduction

Numerous studies indicate that cellular damage caused by reactive oxygen species (ROS) is associated with the incidence of a number of diseases and results of animal studies suggest that oxidative damage may cause adverse effects [1–3]. Therefore, strong attempts have been made over the last decades to identify dietary components with antioxidant properties [4].

Recently, we reported the results of a small intervention trial ($n = 8$) in which we found that consumption of a mix of metal and paper-filtered coffee prevents the endogenous formation of oxidized DNA bases in peripheral human lymphocytes [5]. This is an interesting observation, since coffee is among the most frequently consumed beverages worldwide. Evidence for its antioxidant properties is also supported by results of *in vitro* experiments which showed that it inactivates ROS by direct scavenging [6] and by findings showing induction of antioxidant enzymes in rodents [7, 8]. *In vitro* analyses demonstrated that coffee possesses a higher antioxidant capacity than other

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Abbreviations: 3-NT, 3-nitrotyrosine; **8-iso PGF2α,** 8-isoprostaglandin F2α; **CA,** chlorogenic acid; **DCFH,** 7-dichlorofluorescein; **ENDO III,** endonuclease III; **FPG,** formamidopyrimidine–DNA-glycosylase; **GPx,** glutathione peroxidase; **GSH,** glutathione; **GST,** glutathione-*S*-transferases; **MDA,** malondialdehyde; **oxLDL,** oxidised LDL; **ROS,** reactive oxygen species; **SCGE,** single-cell gel electrophoresis assays; **SOD,** superoxide dismutase; **TAC,** total antioxidant capacity

beverages, *e.g.* fivefold higher than green tea and threefold higher than red wine [9]. Furthermore, it has been stressed that coffee is one of the most important sources of phenolic compounds and that its consumption contributes to a higher extent to the intake of antioxidants than consumption of fruits and vegetables [10]. In this context, it is notable that evidence is increasing that coffee intake is inversely related to the incidence of liver and colon cancer, liver cirrhosis, neurodegenerative disorders and diabetes [6, 11–13]. It is assumed that ROS play aetiology of these diseases. A number of *in vitro* experiments showed that coffee is a rich source of antioxidants and may have beneficial effects against diseases whose pathogenesis involves increased oxidative stress and oxidative damage [1, 2].

Aim of this study was the investigation of the antioxidant properties of an instant coffee enriched in chlorogenic acids (CAs) by co-extraction of water soluble material of a mix of roasted and green beans. CAs are assumed to contribute substantially to the antioxidant properties of the brew [14–16].

It is known that coffee constituents inactivate ROS *via* different molecular mechanisms (for review, see [6]); therefore, we investigated the impact of coffee intake on different groups of macromolecules. The main endpoints (primary outcomes) we studied in this human trial were oxidatively generated DNA lesions, lipid peroxidation and modifications of proteins. Prevention of endogenous formation of oxidized DNA bases was measured in lymphocytes by single-cell gel electrophoresis (SCGE) assays with lesion specific enzymes. 8-Isoprostaglandin F2α (8-iso PGF2α), which is regarded as the most reliable marker of lipid peroxidation [17], was determined in urine and formation of 3-nitrotyrosine (3-NT) was measured in plasma as a marker of functional protein modifications caused by nitric oxide [18]. It is known that these biomarkers are increased in humans with chronic inflammations and by many other diseases [18].

Additionally, a number of other endpoints indicative for antioxidant effects including oxidized LDLs (oxLDLs) and malondialdehyde (MDA) levels in plasma, which are regarded as reliable markers of lipid peroxidation [4] were monitored as secondary outcomes. In addition, further biomarkers of the redox status such as the glutathione (GSH) concentrations in erythrocytes and the total antioxidant capacity (TAC) in plasma were analysed. Since recent findings indicate that coffee might also affect the expression of genes controlled by the transcription factor Nrf2 [7], we measured the impact of coffee consumption on the expression of several genes encoding for cellular antioxidant defense mechanisms by use of real time PCR and by analysis of a panel of antioxidant enzymes activities glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione-*S*-transferases (GST).

2 Materials and methods

2.1 Subjects

The study was approved by the Ethical Commission of the Medical University of Vienna in October 2006 (459/2006) and informed consent was obtained from the participants. Subjects under investigation were healthy adults of both sexes (27 ± 8 years old). The inclusion criteria were BMI: 20–25 kg/m^2, non-smokers, no intake of pharmaceutical drugs, no intake of food supplements 4 wk prior and during the study, no participation in another clinical trial, no pregnancy and no blood withdrawal 3 wk before the study. In total, 36 subjects who fulfilled the inclusion criteria were randomized, 7 subjects were excluded from the *per*-protocol analysis because of adverse events which were seen as likely impairing primary objective of the trial or non-compliance. Among the seven subjects, four subjects had light to moderate diarrhea; one had a urinary tract infection, one a high blood pressure and one was not willing to refrain from coffee drinking during the water drinking period. About 29 subjects (13 men and 16 women) were available for the *per*-protocol analysis. At the beginning and at the end of the trial, blood pressure and the pulse frequencies were monitored. The baseline characteristics for the *per*-protocol population are summarized in Table 1. Two subjects deviate from the BMI inclusion criteria (26 and 27 kg/m^2), and this was seen as a minor protocol deviation and the subjects were analysed.

2.2 Study design

Figure 1 shows the design of the study. One week before the beginning of the intervention phase (coffee/water), the participants were asked to reduce their consumption of fruit juices and other dietary factors such as citrus fruits or cruciferous vegetables, which may have an impact on the outcome of the study (for details, see [19]). Furthermore, they were asked to avoid excessive physical exercise, since it is known that it may cause alteration of DNA migration [20].

The participants were allocated into two groups (18 coffee/water and 18 water/coffee). The individuals in the coffee/water group consumed coffee (800 mL/d) after a 7-day run-in phase; milk and sugar were added *ad libitum*. Following a wash-out period (5 wk) and a restriction phase (1 wk), they drank 800 mL water/d instead of coffee. The participants in the water/coffee group followed the protocol in the reversed order.

All participants were asked to limit their coffee consumption to one cup *per* day 3 wk before the beginning of the run-in phase of the study and during the first 5 wk of the wash-out period.

2.3 Study product

The coffee was provided by the Nestlé Research Center (Lausanne, Switzerland) and distributed in pre-weighed

Table 1. Baseline characteristics of the participants by sex

Gender	Males ($n = 13$)			Females ($n = 16$)		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max
Age (y)	25.2 ± 5.6	20	41	29.3 ± 10.9	20	55
Weight (kg)	76.2 ± 8.6	64	92	61.8 ± 8.1	48	74
Height (cm)	181.7 ± 7.4	173	201	168.2 ± 0.1	157	184
BMI (kg/m^2)	23.0 ± 1.7	20	26	21.8 ± 2.4	19	27
Blood pressure dia (mmHg)	78.8 ± 6.4[a)]	65[a)]	90[a)]	72.9 ± 10.6[a)]	50[a)]	97[a)]
	74.5 ± 14.8[b)]	46[b)]	98[b)]	73.7 ± 8.2[b)]	64[b)]	92[b)]
	81.1 ± 8.9[c)]	67[c)]	97[c)]	75.4 ± 7.2[c)]	67[c)]	90[c)]
Blood pressure sys (mmHg)	132.3 ± 11.4[a)]	115[a)]	154[a)]	118.8 ± 11.4[a)]	105[a)]	154[a)]
	130.2 ± 17.8[b)]	104[b)]	165[b)]	116.9 ± 13.0[b)]	98[b)]	150[b)]
	127.1 ± 14.6[c)]	99[c)]	158[c)]	115.6 ± 13.1[c)]	95[c)]	143[c)]
Pulse frequency (P/min)	67.5 ± 12.8[a)]	42[a)]	89[a)]	76.8 ± 9.9[a)]	56[a)]	96[a)]
	71.7 ± 16.5[b)]	50[b)]	99[b)]	80.7 ± 20.2[b)]	63[b)]	142[b)]
	73.3 ± 20.2[c)]	50[c)]	109[c)]	78.1 ± 13.2[c)]	63[c)]	120[c)]

a) Baseline.
b) Water period.
c) Coffee period.

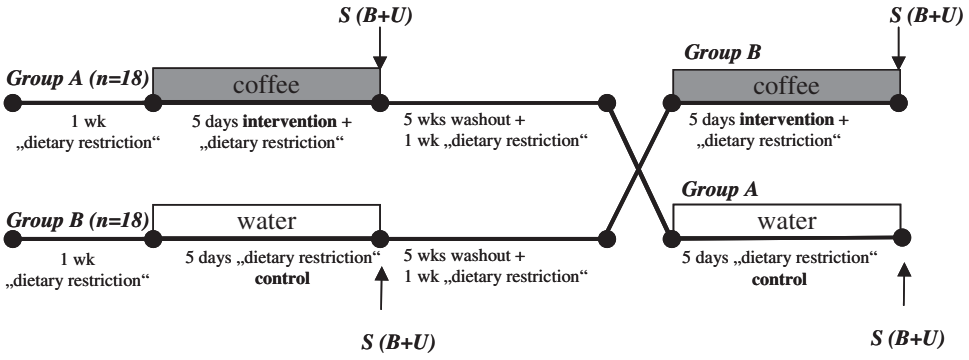

Figure 1. Study design.

portions (3.4 g coffee/package) at the beginning of the intervention. The participants consumed 4 × 200 mL of 1.7% w/w coffee/day without a fixed schedule.

The product was a mix of 35% green and 65% roasted coffee water extracts. Since a large proportion of green coffee antioxidants, in particular CAs, are modified during the roasting process [21], the concentration of CAs was increased by extraction of water soluble material from green (unroasted) beans. A cup of 200 mL coffee contained around 300 mg total CAs; their content was determined as percentage dry basis *via* HPLC analysis (for details, see Table 2).

The analysis of CAs was performed by means of HPLC. Therefore, 1.0 g soluble coffee powder was diluted by addition of 20 mL water and 80 mL methanol. The solution was further diluted tenfold with a methanol–water mix (80:20). The extract was filtered (0.2 µm, FP 30/0.2 CA, Schleicher & Schuell, Basel, Switzerland) and analysed for phenolic acids using a HPLC gradient system (Dionex Ultimate 3000, Sunnyvale, CA, USA) equipped with a binary pump, a degassing system, a UV detector operating at 275 and 325 nm and a Spherisorb column (Waters S5ODS1, 4.6 × 250 mm, PSS839510). Chromatographic data were recorded and integrated with Chromeleon software. A

Table 2. CA content in dry soluble coffee

CA	% Dry basis
3-CQA	1.05
4-CQA	1.24
5-CQA	4.06
4,5-di-CQA	0.58
3,5-di-CQA	0.40
3,4-di-CQA	0.57
4-FQA	0.22
5-FQA	0.79
Total	8.91

gradient consisting of 0.1% v/v phosphoric acid in water/ACN (92:8) as mobile phase A and 0.2 % v/v phosphoric acid in water/ACN (50:50) as mobile phase B was used.

The identification of CGA and derivatives was performed by comparison with the retention times of respective standards and by spiking samples with small amounts of appropriate standards (3-CQA, 4-CQA, 5-CQA, 3.4-di-CQA, 3.5-di-CQA, 4.5-di-CQA (Biopurify, Chengdu, China) and 4-FQA, 5-FQA (synthesized by the Nestlé Research Center)). The quantification of each CGA was performed by comparing the peak areas with those of respective standards.

2.4 Sample preparation

Blood samples were taken by venipuncture after the intervention (coffee or water) on the next day at 8 p.m. after overnight fasting. The last cup of coffee/water was consumed 12–18 h before blood withdrawal. Briefly, 80 mL blood *per* person was collected in heparinized tubes (Becton-Dickinson, Plymouth, UK) and 5 mL blood *per* person was collected in EDTA tubes in order to obtain erythrocytes for GSH measurement.

From each donor, eight heparinized tubes containing 10 mL blood were centrifuged ($650 \times g$, 10 min). Subsequently, plasma was aliquoted in 500 μL portions and stored deep-frozen at −80°C.

Peripheral lymphocytes were isolated with Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) according to the instructions of the manufacturer. Immediately afterwards, lymphocytes were analysed in SCGE assays or stored in liquid nitrogen.

Furthermore, 24-h urine samples were collected, the total amounts were recorded and the samples were aliquoted and stored at −80°C.

2.5 SCGE assays (comet assays)

The SCGE experiments were carried out in peripheral lymphocytes according to international guidelines (23, 24). To avoid variation between results of individual experiments, each of the different parameters was monitored in one experimental series.

Experiments with the DNA lesion-specific enzymes endonuclease III (ENDO III) and formamidopyrimidine–DNA glycosylase (FPG), which enable the detection of endogenous formation of oxidized pyrimidines and purines, were carried out as described by Collins *et al.* [22, 23]. Prior to the main experiment, a calibration experiment was carried out to determine the optimal amounts of the enzymes (data not shown). Lymphocytes (2.5×10^4) were mixed with low melting agarose (0.5%, Gibco, Paisley, UK) and transferred to agarose-coated slides, lysed (4°C, 60 min) and washed (two times with enzyme buffer) prior to incubation with 50 μL of ENDO III or FPG solution for 45 or 30 min, respectively, at 37°C. The solution contained 1.0 μg/mL of the different enzymes. The slides were placed for 40 min in a horizontal electrophoresis unit (C.B.S. Scientific, Del Mar, CA, USA) filled with alkaline solution (pH > 13). Subsequently, electrophoresis was conducted (25 V, 300 mA, 30 min). After neutralisation, the slides were stored at room temperature prior to evaluation (for details, see below).

To monitor alterations of the sensitivity of the lymphocytes towards ROS, the cells were exposed to hydrogen peroxide, H_2O_2 (15 min at 4°C, 25 μM) according to the protocol of Collins *et al.* [24].

After treatment, the cells were mixed with low melting agarose (0.5%, Gibco) and transferred to agarose-coated slides. After lysis (4°C, 60 min) and unwinding in alkaline solution (20 min, pH > 13), electrophoresis was performed (25 V, 300 mA and 20 min).

To determine DNA migration attributable to the treatment with DNA lesion-specific enzymes (Endo III and FPG) and to H_2O_2 treatment, DNA was stained with 40 μL ethidium bromide (20 μg/mL). For each experimental point, three slides were prepared in parallel and from each 50 cells were analysed by a blinded observer with a computer-aided image analysis system (Comet Assay IV Perceptive Instruments, Haverhill, UK). As endpoint, the percentage of DNA in tail was recorded [25, 26]. To calculate the extent of damage attributable to formation of oxidized bases, the extent of DNA migration seen with the respective enzyme buffers was subtracted from the values obtained after exposure of the nuclei to the enzyme buffer solutions. As endpoint, the percentage of DNA in tail was recorded.

Cytotoxic effects were monitored with the trypan blue exclusion technique, which enables the measurement of cell membrane integrity [27]. Only cells from cultures in which the cytotoxic effect was less than 20% were analysed for comet formation [28, 29].

2.6 Determination of lipid peroxidation products

2.6.1 8-Iso PGF2α with LC/MS/MS

8-Iso PGF2α was determined in urine as described Richelle *et al.* [30]. 8-Iso PGF2α was extracted with commercially available immunoaffinity columns (Cayman Chemical, Ellsworth, Ann Arbor, MI, USA) prepared from an 8-iso PGF selective antibody. The procedure was carried out according to the instructions of the manufacturer. After centrifugation ($850 \times g$, 5 min), the urine samples (3.0 mL) were spiked with 5.0 ng of deuterated standard of the title compound, acidified to pH 6.5 with 1 N HCl and passed through a pre-washed immunoaffinity column. After elution with 5.0 mL buffer (95% ethanol/5% water), the eluates were collected and the solvent was evaporated with a fine stream of nitrogen gas. The residue soluble in ACN/water

1/1 (40 μL) was transferred to small siliconized glass inserts in autosampler vials for MSMS analysis.

8-Iso PGF2α was determined with LC/MS/MS (Sciex API 4000 Triple Quad, Applied Biosystems, Van Allen Way, Carlsbad, CA), which involves coupled HPLC (Agilent 1100, Santa Clara, CA, USA) separation of the sample prior to MS analysis. A Zorbax custom made HPLC column of SP-phenyl (Agilent, 3 mm × 50 m, 3.5 μm particle size) was used at a flow rate of 500 μL/min. The sample was injected into the HPLC (20/40 μL total volume) in acetonitrile/water (1:1). The HPLC solvent was programmed from water/acetonitrile in different portions, *i.e.* 2:8 at sample injection to 6.5:3.5 at 0.1 min, and then to 5.5:4.5 at 4.6 min and 0:10 at 6.5 min. The HPLC solvents contained 4 μL propionic acid/litre. The title compound was eluted within 3.2–3.5 min. Selectivity of detection was obtained by analyzing the HPLC eluent using selected ion monitoring (multiple reaction monitoring) for a fragment ion selective for the title compound.

MSMS was operated in ESI mode (negative ionization, −4500 eV and a turbospray ion source maintained at 500°C). MSMS parameters were optimized for each of the deuterated and undeuterated title compounds. We selected the transition *m/z* 353.4–193.1 (MS/MS) for the endogenous compound and *m/z* 357.4–197.1 (MS/MS) for the deuterated internal standard based on mass spectral optimization for each compound. Analysis was carried out using the selective multiple reaction monitoring technique for the above two transitions. Quantitation of the title compound in the urine samples was carried out on the basis of a comparison of the area of the peaks observed for each sample to that of a standard line obtained with a known mixture of undeuterated compound to deuterated internal standard.

2.6.2 MDA

MDA levels were determined in the plasma according to the method of Ramel *et al.* [31] by use of HPLC. Briefly, the samples were hydrolysed after heating (60 min, 100°C) with *o*-phosphoric acid (w/w 85%, Riedel de Haen, Seelze, Germany). This leads to formation of a complex with thiobarbituric acid, which was determined fluorometrically after deproteinization (excitation: λ = 532 nm and emission: λ = 563 nm). Each sample was measured in duplicate.

2.6.3 oxLDLs

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

2.7 Other biochemical parameters

2.7.1 3-NT

The extraction procedure was adapted from Delatour *et al.* [32]. Briefly, 1.6 mL of a cold methanol/acetone mixture was added to 200 μL of plasma to precipitate the proteins. The supernatant was rotary evaporated to dryness. Subsequently, the dry residue was spiked with [d_3]-NTyr (1.51 ng) and [$^{13}C_9$]-Tyr (11.12 μg) and reconstituted in 1.0 mL of ammonium acetate (2.0 mM, pH 4.4) before a two solid-phase extraction steps.

The extract reconstituted in 1.0 mL ammonium acetate (2.0 mM, pH 4.4) was applied onto preconditioned C-18 Bond Elut cartridges (500 mg, 3.0 mL Varian, Middelburg, The Netherlands). The columns were then washed with 1 mL of ammonium acetate 2 mM, pH 4.4, and methanol/ammonium acetate 2 mM, pH 4.4 (5:95 v/v). Tyr and NTyr were eluted with 1.5 mL of methanol/ammonium acetate 2 mM, pH 4.4 (20:80 v/v). The eluates were submitted to a second purification step on aminopropyl cartridges (500 mg, Isolute Separtis, Grellingen, Switzerland). After a conditioning step, the columns were washed with 2.0 mL ammonium acetate (2 mM, pH 3.55) before elution with 1.5 mL of acidic water (pH 1.5). The eluates were dried under vacuum (Speed Vac concentrator from Savant, Farmingdale, NY, USA) and reconstituted in 50 μL of water. The supernatant (*ca.* 40 μL) was then transferred into a HPLC vial for further LC-MS/MS analysis. A total of 10 μL were injected on a HPLC system constituted by a HP series 1100 (Hewlett-Packard, Palo-Alto, CA, USA) and an Xterra MS C_{18} microbore column (150 × 1.0 mm id 3.5 μm, Waters, Milford, MA, USA), operating at a flow rate of 50 μL/min. The separation of NTyr and Tyr was achieved according to the following gradients. Solvent A was water (pH 2.7 adjusted with formic acid) and solvent B methanol/water (pH 2.7, 80:20, v/v); 0–5 min: A/B (100:0); 5–19 min: linear ramp to A/B (37:63); 19–20 min: linear ramp to A/B (0:100), maintained for 4 min.

Detection was performed by positive ESI-MS/MS using a Finnigan MAT TSQ 7000 with API 2 interface (San Jose, CA, USA). Typical retention times were 6.0 min for Tyr and 17.7 min for NTyr. Concerning the source parameters, the spray voltage was set at 2.2 kV, the capillary temperature at 330°C and the sheath gas pressure at 90 psi.

2.7.2 Antioxidant enzymes

CuZn-SOD activity was determined in the cytosolic fractions of lymphocytes with the RANSOD test kit (Randox Laboratories, Ardmore, UK). The test is based on the use of the xanthine/xanthine oxidase reaction to generate O_2^- radicals [33]. The activity of the enzyme was measured spectrophotometrically (λ = 505 nm) by the determination of the degree of inhibition of O_2^--induced formation of a red formazan dye.

GPx activity was determined spectrophotometrically ($\lambda = 340$ nm); the assay is based on the method developed by Gunzler and Flohe [34]. The results were standardized on the basis of the protein concentrations which were measured with the Bradford method [35].

Each measurement was carried out in triplicate.

2.7.3 Intracellular ROS (DCFH-DA assay)

Intracellular ROS levels of peripheral lymphocytes were measured by flow cytometric analysis of 7-dichlorofluorescein (DCFH) oxidation using DCFH-DA (Fluka, Buchs, Switzerland) as described previously [36]. Isolated lymphocytes were stained with PerCP-labeled anti-CD-45 (pan leukocytic marker) and APC-labeled anti-CD-14 (monocyte/macrophage specific) antibodies (BD Pharminogen, San Jose, CA, USA) for 15 min. Afterwards, DCFH-DA (10 μM) was added to the cell suspensions in the presence or absence of autologous plasma (1.0%).

Additionally, lymphocyte suspensions were exposed to 50 μM H_2O_2 (60 min at 37°C). After treatment, the cells were analysed by multiparametric analysis using a FACS Calibur™ system (Becton-Dickinson, San Jose, CA, USA) with excitation and emission settings of 500 ± 15 and 535 ± 15 nm, respectively.

Lymphocytes, monocytes and granulocytes were discriminated by scatter gating strategy (low, moderate and high FSC/SSC, respectively) and CD staining ($CD45^+/CD14^-$, $CD45^+/CD14^+$ and CD45dim/CD14dim).

Background fluorescence was determined in DCFH-delabelled cells without treatment. Each sample was measured in duplicate. Data are given as mean fluorescent peak heights in arbitrary units.

2.7.4 TAC

TAC in plasma was analysed spectrophotometrically (UV/VIS Spectrometer Lamda2; Perkin Elmer,, Waltham, MA, USA; $\lambda = 734$ nm) after iron-induced oxidation. The assay is based on the ability of an antioxidant to scavenge blue–green-colored 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radicals ($ABTS^*$) which are generated by the peroxidase activity of metmyoglobin in the presence of the sample [37].

2.7.5 GSH

GSH measurements were carried out immediately after taking blood. GSH levels were determined photometrically in erythrocytes. The tripeptide was released from the cells by hemolysis [38], which leads to tehe removal of protein and blood pigments. The remaining suspensions were mixed with 5,5′-dithiobis-2-nitrobenzoic acid and absorbance read at 412 nm (UV/VIS Photometer ATI-Unicam UV 4, Perkin Elmer). The measurements were carried out in duplicate.

2.7.6 GST

GST activity was spectrophotometrically ($\lambda = 340$ nm) determined in the cytosols of lymphocytes according to the standard method developed by Habig *et al.* [39] at 37°C, using 1-chloro-2,4-dinitrobenzene as a substrate. The protein contents were determined with the BIORAD/Bradford assay [35]. All measurements were carried out in triplicate.

2.7.7 Gene expression by low-density array

For RNA preparation, the lymphocytes were separated with cell preparation tubes (Vacutainer, Becton Dickinson, Plymouth, UK). Subsequently, the pellets were treated with trizol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Samples were stored at –80°C. RNA was converted to cDNA using the TaqMan reverse transcription kit system (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA), using the random hexamers primer and 2.0 μg of pooled RNA samples. cDNA synthesis was performed in a thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min at 25°C, 30 min at 48°C and a final inactivation step of 5 min at 95°C. Samples were amplified with Taqman low-density custom arrays with the ABI Prism 7900HT sequence Detection System using 1.0 μL of cDNA template and the TaqMan PCR master mix 2 × (Applied Biosystems, Foster City, CA, USA). The thermocycler conditions included two incubations (2 min at 50°C and 10 min at 95°C), followed by 40 cycles, each consisting of a denaturation step for 15 s at 95°C and a second annealing and extension step for 1 min at 60°C. Quantification of amplified PCR products was performed using ABI Prism®7900HT Sequence Detection System software Version 2.2. (Applied Biosystems, Foster City, CA, USA) and normalized to the glyceraldehyde 3-phosphate dehydrogenase gene as an internal control. All PCR probes and primers used in this study, NAD(P)H dehydrogenase quinone oxidoreductase 1 (NQO1), SOD1, UDP glucuronosyltransferase 2 family (UGT 2B1, 2B3, 2B15, 2B17), GST subunits (A1, A2, A5, T1 and T2), were obtained from the low-density custom array of Applied Biosystems.

2.8 Statistical analyses

2.8.1 Confirmatory part

The calculation of sample size for % DNA in tail was based on background knowledge from the literature data. It was

concluded that a 15% difference should be detectable with $n = 32$. For 8-iso prostaglandin F2α according to Dillon *et al.* [40], the mean basal value in nonsmokers is 272 pmol/mmol creatinine. These authors are displaying a *p*-value of 0.23; from this *p*-value, we estimated the intra-individual standard deviation with 62 pmol/mmol. With an α-level of 0.05/4, four comparisons (Bonferroni adjustment) and a power of 80%, we need 32 complete subjects to detect a difference of 25%.

For 3-NT in plasma, no background knowledge was provided. However, a difference of 10% was regarded as relevant.

The following statistical analysis was planned in advance for the primary outcomes: Wilcoxon signed rank tests for 8-iso prostaglandin F2α, Comet ENDO III median % tail intensity and Comet FPG median % tail intensity. 3-NT in plasma was analysed by a mixed model correcting for period. The *p*-values were adjusted for multiplicity according to Hommel; the simultaneous 95% confidence intervals were adjusted according to Bonferroni. Therefore, the confidence intervals are not always in agreement with the *p*-values.

The statistical analysis described above indicated that the four primary outcomes were pointing in coherent directions, suggesting that they are positively correlated. This motivated the application of a further global statistical test. Global statistical tests are providing inference on several outcomes together and not outcome-by-outcome as univariate tests do. O'Brien suggested [41] a test that assumes that all outcomes are positively correlated. This approach has some undesirable mathematical properties, which were fixed by Tang in 1993 [42]. This extended version was regarded as suitable for the analysis of the markers of oxidative stress and DNA damage. In order to have a small sample approximation of the test statistic, the null distribution was estimated by bootstrapping. The test was programmed with the help of *R* and validated along an example provided in the Tang publication. Additional to the global statistical test, the *p*-values of the individual comparisons were adjusted for multiplicity according to closed testing. The intersection hypotheses were also tested by the O'Brien's test. These procedures are controlling false-positive rates on the 5% level and provide evidence on primary outcomes.

2.8.2 Exploratory part

Secondary outcomes were analysed by a mixed model correcting for period. Residuals were checked for normality by qq-plots. If departure of normality was observed, log-transformation (basis e) was applied. If still after log-transformation departure of normality was observed, non-parametric techniques were applied. Paired observations were analysed by Wilcoxon signed rank tests; treatment differences were displayed by Hodges–Lehmann (pseudo) medians and 95% confidence intervals [43]. The *p*-values displayed for secondary outcomes are not corrected for multiplicity and may be interpreted as flags, indicating interesting results.

3 Results

The impact of coffee consumption on primary outcomes is summarized in Table 3. After 5 days of coffee consumption (four cups *per* day), the individual levels of the four biomarkers of macromolecular oxidative damage selected as primary outcome of the study were lower as compared with controls. In order to evaluate the overall evidence of changes of the four primary outcomes, the O'Brien test was applied. The impact of coffee consumption on the biomarkers as considered together was found to be highly significant ($p = 0.0016$), suggesting protection against oxidative damage. Lipid peroxidation as measured by 8-iso PGF2α in urine and protein damage measured by 3-NT level in plasma were decreased by 15.3 and 16.1%, respectively. The adjusted *p*-values (according to the close testing method for multiple tests) indicated that urinary isoprostane ($p = 0.022$) and 3-NT ($p = 0.027$) were significantly decreased after coffee intake. Similarly, DNA migration attributable to FPG and ENDO III sensitive sites (reflecting endogenous formation of oxidized purines and pyrimidines) in lymphocytes was reduced (by 14.1 and 12.5%, respectively). However, these effects did not reach significance.

The secondary outcomes are listed in Table 4. Several markers of the oxidant status were altered at the end of the intervention study. The most pronounced effect was the reduction of intracellular ROS by 20.3% after exposure of intact cells to hydrogen peroxide in the presence of autologous plasma. All other biological effects were only moderate and none of them reached significance. However, it is notable that coffee intake shifted several endpoints towards a reduction of oxidative damage. The level of both lipid peroxidation products (oxLDL and MDA) was slightly reduced after coffee consumption (5 and 8.1%, respectively) and a small decrease (about 5%) of DNA damage in SCGE experiments was observed after treatment of the nuclei with ROS (H_2O_2).

Furthermore, we analysed the expression of 30 genes involved in the cellular antioxidant defense by RT-PCR (low-density array) and no significant differences attributable to coffee consumption were observed (data not shown). However, some of them *NQO1* (7.8%), *SOD1* (22.2%), *UGT2A1* (125.4%), *UGT2A3* (14.4%), *UGT2B15* (20.6%), *UGT2B17* (15.0%), *GSTA1* (24.8%), *GSTA2* (45%), *GSTA5* (19.5%), *GSTT1* (19.5%) and *GSTT2* (19.5%) were increased after coffee consumption (numbers in parenthesis indicate the increase in percent after coffee consumption). In addition, the expression of the transcription factor Nrf2 which is known to mediate the induction of several of these genes was increased (9.9%). However, due to substantial

Table 3. Impact of coffee consumption on parameters of oxidative stress defined as primary outcome variables

Endpoints	Water period[a)]	Coffee period[a)]	Δ (%)[b)]	95% CI (%)[c)]	*p*-Value[d)]	GT[e)]
ENDO III (% DNA in tail)	9.91±3.42	8.70±4.00	−14.1	−36.1, 9.5	0.138	0.0016
Formamido pyrimidine glycosylase (% DNA in tail)	15.7±6.98	14.07±5.32	−12.5	−46.9, 22.2	0.417	
3-NT (pg/μg tyrosine)	8.47±3.09	7.01±2.38	−16.1	−34.5, 2.3	0.027	
8-iso PGF2α/creatinine (pg/mg)	86.7±41.4	74.85±34.78	−15.3	−30.7, 0.3	0.022	

CI, confidence interval.
a) Mean±SD, $n = 29$, except for ENDO III ($n = 27$).
b) Calculated in % of the means of the water period.
c) Simultaneous 95% confidence intervals according to Bonferroni.
d) Adjusted according to closed testing.
e) GT, global statistical test and *p*-value according to O'Brien.

Table 4. Impact of coffee consumption on endpoints of oxidative stress defined as secondary outcome variables

Parameters (unit)	Water period[a)]	Coffee period[a)]	Δ (%)[b)]	*p*-Value[c)]
Lipid peroxidation products				
oxLDL (U/L)	40.05±11.41	38.05±13.48	−5.0	0.31
MDA (μM/L)	1.16±0.68	1.07±0.59	−8.1	0.35
Enzymes				
SOD (U/mg protein)	7.60±2.66	7.32±2.40	−2.7	0.66
GPx (U/mg protein)	0.56±0.19	0.58±0.26	+1.7	0.52
GST (nmol/min/mg protein)	277.58±86.90	288.36±78.38	+4.0	0.35
Intracellular ROS (arbitrary units)				
Without challenge (−plasma)	10.62±4.17	10.28±2.67	−3.5	0.48
With H_2O_2 (−plasma)	151.89±92.74	166.92±99.84	+14.5	0.41
With H_2O_2 (+plasma)	57.06±42.84	42.96±30.44	−20.3	0.27
SCGE assay (% DNA in tail)				
H_2O_2 induced DNA damage	17.8±11.5	17.04±9.34	−4.9	0.36
Other parameters				
TAC (mM TE)	0.71±0.16	0.76±0.15	+5.2	0.23
GSH (mg/dL)	72.52±9.42	72.43±11.28	−0.37	0.77

TE, trolox equivalent.
a) Mean±SD, measurements were carried out in lymphocytes, except the determinations of oxLDL, MDA, TAC, which were carried out by using plasma, and GSH which was measured in erythrocytes.
b) Calculated in % of the means of the water period.
c) Statistical analyses were performed with the Wilcoxon signed rank test and treatment differences were displayed by Hodges–Lehmann and 95% confidence intervals.

inter-individuals variability, none of these effects reached statistical significance.

4 Discussion

Overall, the results of this study indicate that consumption of instant coffee containing increased levels of CAs causes protection of certain macromolecules (*i.e.* of cell lipids in membranes and proteins) against oxidative damage in healthy adults.

The study design was based on the evaluation of the parameters of the previous intervention trials in which the SCGE technique was used [19]. The number of participants in 80% of the previous trails was lower than that we recruited in this study. The intervention time (5 days) of this study was the same as in a previous smaller trial with coffee [5] in which pronounced effects were found. It is also notable that results from animal experiments indicate that this is a sufficient time for the induction of transcription factors and enzymes by coffee constituents [7, 44].

The reduction of oxidatively generated DNA lesions was not unexpected, as we found a clear decrease of FPG and ENDO III sensitive lesions in a recent pilot study with coffee [5]. The participants consumed a mix of paper and metal-filtered coffee in the aforementioned trial, which contained relatively high levels of diterpenoids in contrast to the instant coffee, which was used in the present trial. This difference may explain the reason why we found qualitatively similar although quantitatively less-pronounced effects in our study. Furthermore, our data support the findings of a number of earlier studies that are indicative for

the prevention of oxidative DNA damage by coffee. For example, van Zeeland *et al.* [45] reported on reduced level of 8-hydroxydeoxyguanosine in peripheral leucocytes of coffee drinkers and it was found in several *in vitro* experiments that coffee and also some of its constituents such as caffeine, Maillard products, trigonelline and CAs possess antioxidant properties and protect cells against ROS-induced DNA damage (for review, see [6]).

As described above, we also observed a decrease of the F2-isoprostane (8-iso PGF2α) levels in urine by 15.3% in the present trial ($p = 0.022$). Isoprostanes are regarded as a reliable maker of lipid peroxidation [17] and high levels are associated with increased risks of a number of diseases including neurological disorders, coronary heart and lung diseases and renal dysfunction to name a few (for review, see [46]). Moreover, the assumption of a protective effect is supported by the results of a number of *in vitro* experiments with coffee in which a reduction of lipid peroxidation products was observed [47, 48], whereas no changes of plasma F2-isoprostanes levels were found in a recent study by Mursu *et al.* [49] who investigated the effects of coffee in a large human intervention trial. However, a different type of coffee (*i.e.* paper-filtered ground coffee) was tested in this study and isoprostanes were monitored in plasma and not in urine.

A further primary marker which we measured was the 3-NT level in plasma which is indicative for pathological conditions such as inflammation, neurodegenerative diseases, cardiovascular disorders and atherosclerotic lesions [18]. Our study shows that coffee consumption reduces the 3-NT levels substantially *i.e.* by 16.1% ($p = 0.027$). This finding is in agreement with the results of *in vitro* experiments which showed that hydroxycinnamic acids inactivate peroxynitrite [50], which is responsible for the nitration of tyrosine [51]. Furthermore, it has been shown in rats that CA reduces the NT levels in blood vessels; this effect was paralleled by reduction of hypertension and improvement of endothelial function [52].

In the RT-PCR (low-density array) experiments, a moderate increase of the expression of some genes encoding for enzymes involved in antioxidant defense and detoxification such as NQO, SOD1 and specific forms of UGT and GST were found. None of these changes was significant; nevertheless, they suggest a trend towards a specific pattern of transcriptional changes. Cavin *et al.* [7] found in *in vitro* and also in mice that coffee and coffee-specific diterpenoids activate the transcription factor Nrf2 which controls the aforementioned genes and it is notable that we detected a moderate (not significant) activation of the expression of this factor in the lymphocytes in the present investigation.

None of the other markers (GSH, MDA, oxLDL, SOD, GPx and intracellular ROS formation) was altered significantly after coffee intake. These findings are partly in contrast to the results of earlier studies. For example, Esposito *et al.* [53] found increased GSH levels in an Italian study after consumption of espresso coffee; Bichler *et al.* [5] detected an increase of SOD activities with a mix of metal and paper-filtered coffee and Steinkellner *et al.* [54] found a strong enhancement of the levels of GST isoenzymes with metal-filtered coffee. On the contrary, no changes of these markers were found by Mursu *et al.* [49], who conducted trials with paper-filtered ground coffee containing low amounts of cafestol and kahweol. Nardini *et al.* [55] detected a significant increase of the plasma antioxidant capacity in humans 60 min after consumption of 200 mL of brewed coffee. In our study, TAC analysis was performed overnight after the last coffee intake and only a slight increase was observed. However, recent data on the bioavailability of CAs showed that their absorption is biphasic with maximal plasma concentration observed much earlier (around 2 and 8 h) [56–58].

As described above, the protective effects of the coffee brand we tested may be due to its high levels of CAs as many *in vitro* and animal studies showed that they are potential antioxidants; nevertheless, coffee also contains other bioactive components such as Maillard products, aminoreductone and *N*-methylpyridinum. To elucidate the role of CAs assumption, we plan to conduct a comparative trial with different coffee types in which we will focus on the endpoints which were altered in this study. In this context, it is notable that Bakuradze *et al.* [59] reported recently on a small trial (results are only available in abstract form) in which they compared the prevention of oxidative DNA damage caused by two types of coffee, one with an increased level of CA and one with high level of *N*-methylpyridinium. The most pronounced protection was found after intervention with CA-rich coffee.

The differences in the responsivity of the different endpoints between the different coffee studies are probably caused by use of different brews and by the multiple mechanisms which account for the antioxidant properties of coffees and their constituents. They include direct scavenging of free radicals as well as induction of protective enzymes (*via* interactions with transcription factors (for review, see [6]). Furthermore, also reduction of glucose absorption, which was found in earlier human trials with instant coffee containing increased concentrations of hydroxycinnamic acids, may play a role [60]. A similar effect was also found with green tea and it has been emphasized that the hypocaloric status resulting from reduced sugar uptake may alter mitochondrial functions and cause decreased ROS production. This indirect antioxidant effect is not paralleled by alterations of the antioxidant status in plasma [61].

Dotan *et al.* [62] found strong discrepancies in a recent evaluation of the correlations of alterations of antioxidant parameters in human and animal studies with dietary components. The lack of associations between different clusters of endpoints shows that no individual general maker for protection against oxidative damage can be defined. The validation of the reliability of currently used methods for the identification of dietary factors led us to the

conclusion that combinations of different biomarkers should be used to draw firm conclusion and that isoprostane levels in plasma and urine and markers of oxidative DNA damage are probably the most relevant ones as the methods are robust and their relationship to the aetiology of human diseases is well established [4]. This holds true also for the 3-NT levels in plasma [18]. One of the limitations encountered in most antioxidant human studies is that the different endpoints can be measured only in body fluids and in blood cells but not in inner organs and that they provide only indirect evidence for protection. In this context, it is notable that we found recently with gallic acid, which is an extremely potent antioxidant in lymphocytes in humans, that this phenolic also causes pronounced protection in many inner organs of rodents [63]; however, it is unclear if such effects can also be expected with coffee.

As described above, the overall evaluation of the alterations of these biomarkers which were selected as primary outcome of the study shows that coffee intake causes significant protective effect against damage mediated by free radicals, indicating that it may have beneficial health effects in human in regard to prevention of ROS-associated diseases.

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