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Dark coffee consumption protects human blood cells from spontaneous DNA damage

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ABSTRACT

Coffee increasingly attracts notice with respect to beneficial health effects. Our objective was to investigate DNA protective effects of a special roast coffee blend of pure Arabica (*Coffea arabica* L.) in healthy volunteers (n = 96), following a prospective, randomized, controlled study with parallel design (coffee versus water). Potential modulation of Nrf2 signaling was evaluated by focusing on its two master regulators, Nrf2 and Keap1, as well as on Nrf2 translocation in the volunteers' lymphocytes (PBLs). In this context a newly established fluorescence imaging method for Nrf2 translocation analysis in PBLs turned out as feasible and eligible tool applicable for future studies.

After chronical coffee consumption (8 weeks) spontaneous DNA strand breaks were significantly lower in the coffee group compared to water control, suggesting a protective effect of the coffee blend. Nrf2 signaling was remotely affected, indicating that additional mechanisms of protection from DNA damage need to be considered.

1. Introduction

Coffee and its constituents have been increasingly studied with respect to potentially beneficial as well as adverse health effects. The most investigated constituents of coffee beverage are alkaloids (caffeine and trigonelline), phenolic compounds (chlorogenic acids), and diterpenes (cafestol and kahweol). They are considered as potential antioxidants and partly described as free radical scavengers (Lee & Jeong, 2007; Yashin, Yashin, Wang, & Nemzer, 2013).

Numerous epidemiological and *in vitro* data suggest that coffee consumption is associated with a reduced risk of certain cancers, cardiovascular diseases, diabetes and inflammatory bowel diseases (Bidel & Tuomilehto, 2013; Ding et al., 2015; Loftfield et al., 2018; Malerba et al., 2013; Poole et al., 2017; von Ruesten, Feller, Bergmann, & Boeing, 2013). Many of these diseases are associated with an impairment of DNA integrity potentially due to increased oxidative stress. Data from several *in vitro* and *in vivo* studies already suggested antioxidant and other DNA-protective effects of coffee constituents with different chemical compositions (Bakuradze et al., 2010; Bichler et al., 2007; Esposito et al., 2003; Hoelzl et al., 2010; Misik et al., 2010). As potential mode of action, a cellular status induced by coffee constituents might be hypothesized, leading to the protection of DNA against oxidants and toxic compounds. In this context special attention was given to the master regulator of oxidative stress, the transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) and its signaling pathway. The major function of Nrf2 is the activation of the antioxidant response element (ARE) thereby triggering the transcription of a wide variety of genes that are capable to protect the organism against xenobiotics and/or oxidative stress. Thus, Nrf2 represents one of the main cell defense mechanisms and major regulator of cell survival (Jeong, Jun, & Kong, 2006; Kensler, Wakabayashi, & Biswal, 2007; Lau, Villeneuve, Sun, Wong, & Zhang, 2008; Zhang, 2006). Under quiescent conditions Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytosol and is in homeostasis by basal biosynthesis and

Abbreviations: ARE/EpRE, antioxidant/electrophile response elements; PBL, peripheral blood lymphocytes; ITT, intention to treat; Keap1, Kelch-like ECH-associated protein 1; KEAP1, gene name of Keap1; Nrf2, nuclear factor erythroid 2 (NFE2)-related factor 2; NFE2L2, gene name of Nrf2; PP, per protocol; R-factor, ratio between immune- fluorescence signals of nucleus and whole cell; RQ, relative quantity

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Table 1
Specifications and contents of characteristic coffee compounds of the *Arabica* roast coffee blend C21.

Coffee constituent	C21 Coffee content specifications [mg/g]	Specification limits [mg/g]	Actual contents in coffee applied [mg/g]	Maximum ranges occurring during industrial production [mg/g]
Caffeoylquinic acids[a]	10.18 (±10% dw)	9.16 – 11.20	9.37	9.30 – 9.50
N-Methyl-pyridinium	1.10 (±10% dw)	0.99 – 1.21	1.12	1.11 – 1.13
Trigonelline[b]	3.82 (+10% dw)	up to 4.20	4.00	4.00 – 4.01

[a]Sum of 3-, 4- and 5-CQA.
[b]The trigonelline content should be regarded as the upper limit with proven efficacy. Trigonelline has been reported to interfere with the induction of protective cellular responses by coffee (Boettler, Volz et al., 2011). This limit refers to the content of trigonelline in coffee as used in a previous trial by Bakuradze and colleagues (Bakuradze et al., 2015), including a plus of 10.0% dw to accommodate for variations in coffee roasting, preparation and analytics. ± SD of four independent measurements, dw = dry weight.

proteasomal degradation. Under activating conditions (oxidants, electrophiles, ROS) Nrf2 is released, translocated into the nucleus and bound in complex with other factors to antioxidant or electrophile response elements (ARE/EpRE). The ARE/EpREs are located in the promoter regions of several cell defense genes, comprising among others several phase II detoxifying enzymes (glutathione-S-transferases (GSTs), γ-glutamylcysteine-ligase, etc.) and enzymes involved in antioxidant defense such as haemoxygenase-1 (HO-1) or NAD(P)H dehydrogenase [quinone] 1 (NQO1). Consequently, there is increased expression of such protective enzymes in the cell, generating a state of improved defense against oxidative stress (Tebay et al., 2015).

Recent *in vitro* studies have shown that exposure to coffee extracts of different origin as well as selected constituents (5-*O*-caffeoylquinic acid, pyridinium derivatives such as N-methylpyridiniumion (NMP)) caused nuclear translocation of Nrf2 and induction of phase II antioxidant enzymes in HT-29 human colon carcinoma cells (Boettler, Sommerfeld et al., 2011; Volz et al., 2012). Paur and colleagues demonstrated that the degree of roasting determines the efficiency of improving inflammation-induced NF-κB activity and the induction of antioxidant defense via Nrf2/ARE signaling *in vitro* and *in vivo* (Paur, Balstad, & Blomhoff, 2010). Fine-tuning of the degradation/formation of Nrf2/ARE-activating and -deactivating compounds during the roasting process appeared crucial for the protective properties of the coffee product (Bakuradze et al., 2010; Boettler, Sommerfeld et al., 2011). When comparing caffeic acid, catechol, hydroxyhydroquinone, trigonelline and the alkylpyridinium compounds by far the strongest Nrf2-inducing effects were observed for the alkylpyridinium NMP (a degradation product of trigonelline during roasting) (Boettler, Volz et al., 2011). This might be linked to their strong antioxidant activity *in vivo* (Somoza et al., 2003) through Nrf2 translocation (Boettler, Volz et al., 2011; Boettler, Sommerfeld et al., 2011). These results, *in vitro* and in mammals, provide a plausible mechanism for improved DNA integrity after consumption of coffee. Furthermore, several recently published human intervention studies point to the protective effect of coffee consumption on background DNA damage (Bakuradze et al., 2011, 2015, 2016, 2014).

Main objective of the present intervention study was to investigate DNA protective effects of a well-defined dark roast coffee blend of pure *Arabica* (*Coffea arabica* L.) in healthy male and female volunteers by measuring DNA strand breaks in blood cells with the comet assay. The assay is considered as a useful tool in human biomonitoring of DNA damage (Collins et al., 2014; Dusinska & Collins, 2008; Speit & Rothfuss, 2012; Valverde & Rojas, 2009). The composition of the coffee blend was optimized with respect to consumers' acceptance, taste and most effective health benefit adapted from previous results in the literature (Bakuradze et al., 2011, 2015; Bakuradze et al., 2014; Boettler, Volz et al., 2011; Riedel, 2014; Volz et al., 2012). We hypothesized that the level of spontaneous DNA damage in the blood cells of healthy volunteers will be reduced by consumption of the coffee blend over a prolonged time-period (eight weeks) as compared to the control group consuming water instead. Furthermore, the potential mechanism of DNA-protection, i.e. the modulation of Nrf2 signaling, was evaluated by focusing on the impact of the coffee blend on the transcription of the two master regulators, Nrf2 and Keap1, as well as on Nrf2-translocation in the volunteers' lymphocytes, applying a newly established fluorescence imaging method.

2. Material and methods

2.1. Specification and preparation of study coffee

The study coffee (named coffee C21) was a blend of pure *Arabica* roast coffee (*Coffea arabica* L.) produced by Tchibo GmbH, Hamburg, Germany. The present coffee blend was industrially manufactured in an identical way as reported in a previous study (Bakuradze et al., 2015). The initial specifications of characteristic roast-dependent coffee compounds of the coffee blend, their actual content, and their maximum deviations during industrial production are listed in Table 1.

Quantitation of trigonelline and N-methylpyridinium was carried out using a stable isotope dilution based HILIC-LC-MS/MS method (Lang et al., 2013). Analyses were performed by a laboratory, accredited for this method (Eurofins Analytik GmbH, Hamburg, Germany). Caffeoyl quinic acids and caffeine were determined by another accredited laboratory (CR3-Kaffeeveredelung M. Hermsen GmbH, Bremen, Germany) applying the respective (HPLC-UV) DIN procedures (DIN 10767:2015-08, DIN ISO 20481/ASU 46.00-3). In contrast to the characterizing compounds in Table 1, the relative caffeine content is hardly roast-dependent (Casal, Oliveira, & Ferreira, 2000). Being the most prominent bio-active of coffee, caffeine was quantified nevertheless. The actual caffeine content of the coffee blend amounted to 6.69 mg/g in dried powder. Assuming 100% caffeine transfer from each coffee pad to the beverage, the daily consumption of six pads of the actual coffee applied, accordingly would sum up to 301 mg caffeine. Hence, the individual caffeine consumption per day within the coffee group was consistent with EFSA caffeine safety evaluation, i.e. it fell below 400 mg/day (Scientific Opinion on the Safety of Caffeine, 2015).

For consumption, the coffee beverage was freshly prepared with a common Senseo®-type pad-machine. The quantity of the coffee was 3 large cups (250 mL/cup) per day, brewed from 6 pads (3 × 2), each filled with 7.5 g (± 0.1 g) of coffee, and extracted with tap water to achieve 250 mL of brewed beverage.

2.2. Subjects and study design

The study had a single centered, controlled, randomized, parallel study design starting with a "run-in" period (week 1–4) and ending with the intervention period (week 5–12). The study was approved by the local ethics committee of the University of Vienna (Reference Number: 00202, 22nd September 2016) and conducted by the group of V. Somoza (Department of Physiological Chemistry, Faculty of Chemistry, University of Vienna). The volunteers have been informed elaborately about the study procedure and gave their written informed consent for participation. All applicants underwent baseline medical examinations (including blood sample analysis) and anthropometric measurements.

They were healthy, non-smoking, central European male and female volunteers (aged 19–50 years, body mass index (BMI) 19–32 kg/m^2) and accustomed to the daily consumption of coffee. Exclusion criteria were excessive sports, frequent intake of pharmaceutical drugs (except oral contraceptives) or food supplements, metabolic disorders, participation in blood donation or in other studies, pregnancy, alcohol abuse, being under permanent medical treatment.

One hundred and thirty-six individuals have been recruited, of which finally 96 volunteers fully participated in the 12 weeks intervention study, from 30th January to 7th April 2017. They were randomly allocated (stratification according to the BMI) to two groups after the "run-in" phase: intervention/coffee group with n = 48 (23♂, 25♀) and control/water group with n = 48, (23♂, 25♀), thus each consisting of an equal number of men and women. During the 4 weeks "run-in" period both groups consumed a minimum of 750 mL of water daily, in three equal portions (morning, noontime, afternoon). During the 8 weeks intervention period, one group daily consumed 750 mL of freshly prepared coffee beverage (coffee group) and the other group (control group) instead the same volume of warm water. During the entire study period, the subjects refrained from all coffee beverages (except study coffee for the intervention group in the intervention phase), tea (black, green, white), and caffeine drinks. They were asked to consume only in minor amounts polyphenol-rich food and drink, like: red wine, cocoa, malt coffee, dark chocolate and berry juices, as well as any intensely colored fruits and vegetables or juices that were not listed on the provided information sheet regarding allowed foods and drinks. The dietary regime considered a limited intake of polyphenols. Overall, the dietary guidelines provided to the study subjects were intended to smoothen the background modulatory effects of the diet on Nrf2 activity which may occur in individuals consuming high amounts of polyphenol-rich foods or drinks (except study coffee). Moreover, participants were asked to refrain from nutritional supplements (such as vitamin supplements, extracts) and from multi-vitamin juice. Apart from that they followed their usual dietary habits. In the last week of the run-in period and intervention period, study participants had to document their food intake for a time period of seven days, for detail see Hochkogler et al., 2019

After the four weeks "run-in" period venous blood samples were taken (V1 = visit 1; both groups) two hours after 250 mL water intake and on the last day of the intervention period (V2 = visit 2) two hours after coffee (coffee group) or water (control group) intake (each 250 mL), respectively. At each visit 20 mL blood were taken and 50 mL urine were collected for monitoring compliance during the study (Hochkogler et al., 2019). Blood and urine samples were blinded during analyses by the supervisor of the study.

2.3. Analysis of spontaneous DNA strand breaks (comet assay)

Single cell gel electrophoresis (comet assay) under alkaline conditions was performed according to Collins (2014) following the protocol of Bakuradze et al. with minor modification (Bakuradze et al., 2015). Whole blood was used in the comet assay without preceding isolation of lymphocytes assuring minimal cell damage during processing. Venous blood samples were collected in EDTA-coated tubes (4–5 mL). An aliquot of EDTA-blood (6 µL) was mixed with low melting agarose (0.7%, 65 µL; 2 gel-pads/slide) directly after the blood draw (not later than 30 min, room temperature). Subsequently, the cell suspension was distributed on a frosted glass slide pre-coated with a layer of normal melting agarose (0.5%) and cover-slipped. Slides were coded for blinding by beforehand engraving. After solidification on ice (10 min) the cover slips were removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris pH 10, 1% laurylsarcrosin, 10% DMSO, 1% Triton X) for 2 h at 4 °C. For every set of samples processed (1 set per day), a positive control was included, consisting of embedded blood cells of one randomly chosen participant of the respective set. These cells were irradiated for 1 min with UV-B light (λ = 312 nm; energy: 1 J), inducing DNA strand-breaks. Then cells were replaced in lysis buffer. Subsequently slides were prepared for gel electrophoresis by equilibrating in electrophoresis buffer (60 mL 10 M NaOH, 10 mL 200 mM EDTA pH10, add 2 L) for 20 min at 4 °C in the dark allowing DNA to unwind. Horizontal gel electrophoresis was performed at 4 °C for 20 min (25 V, 300 mA). Then slides were washed in neutralization buffer (0.4 M Tris/HCl, pH 7.5) three times for 5 min at 4 °C and stored over night at 4 °C. Next day cells were stained with ethidium bromide (20 µg/mL) and analyzed fluorescence microscopically with a Zeiss Axioskop 20 FL (λex = 546 ± 12 nm; λem ≥ 590 nm) at a 20-fold magnification. Slides were subjected to computer-aided image analysis (Comet Assay IV System, Perceptive Instruments, Suffolk, Great Britain), scoring 200 randomly picked cells per slide. The intensity of DNA in the comet tail was quantified and calculated as percentage of overall DNA intensity in the respective cell (TI [%]).

2.4. Preparation of human lymphocytes

Freshly collected human blood (5 mL) anticoagulated with EDTA was layered on 7 mL Histopaque 1077 (Sigma Aldrich). After centrifugation for 30 min (400 rcf, without break, 25 °C) the lymphocytes were collected from the layer between the plasma and Histopaque 1077 phases and were transferred into 10 mL of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life Technologies). Subsequently, the cell suspension was centrifuged for 10 min (350 rcf, 25 °C), and the cell pellet was resolved in 5 mL of phosphate-buffered saline (DPBS, Fisher Scientific). Lymphocytes were repeatedly washed in DPBS and centrifuged. Cells were transferred either into 0.6 mL of RNAprotect Cell Reagent (Qiagen) for subsequent transcription analysis or in 750 µL freezing solution (66.7% RPMI 1640 medium (Gibco, Life Technologies), 23.3% FBS, 10% DMSO) for storage at −80 °C and future immune-fluorescence imaging analysis.

2.5. Analysis of Nrf2 translocation by immune-fluorescence imaging

The analysis of Nrf2 translocation was assessed by a highly sensitive immune-fluorescence imaging approach, enabling simultaneously detection of Nrf2 and phosphorylated Nrf2 protein in the nucleus and the whole cell. The protocol relied on the method described in Jarolim et al. (2017) for adherent HT29 colon tumor cells and was adapted with some modifications to human PBLs.

In detail, frozen lymphocytes obtained in visit 1 and 2 from volunteers of the intervention study were thawed and washed 2 times in 5 mL DPBS (1200 rcf, 3 min). Cell pellet was re-suspended in 300 µL fixing solution (90 mL PBS A = 0.2 g KH_2PO_4, 0.2 g KCl, 9 g NaCl, 2.2 g NaH_2PO_4 add 500 mL, pH 7.2 plus 10 mL 37.5% formaldehyde) and incubated at room temperature for 10 min followed by a second centrifugation step (1200 rcf, 3 min). Cells from visit 1 and 2 of each volunteer were re-suspended in 300 µL DPBS and evenly distributed in 2–3 wells of a 96-well plate respectively (Corning, black side and clear bottom). Cells were controlled microscopically regarding density, agglomeration, debris and presence of monocytes. Then the tissue culture

plate was centrifuged at 1200 rcf for 3 min to pellet the cells. Supernatant was soaked off and cells were permeabilized by adding 100 µL permeabilization buffer (100 mL PBS A plus 200 µL Triton X) per well and incubating for 5 min at room temperature. Another centrifugation step (1200 rcf, 3 min) followed and cells were subsequently washed once with DPBS. Next, 100 µL/well blocking solution (100 mL PBS A plus 0.75 g glycine, 5% goat serum) was added and cells were incubated for 1 h at room temperature. Thereafter cells were centrifuged (1200 rcf, 3 min) and the blocking solution removed. For cell staining a mixture of Nrf2 (anti NRF2 antibody 89,443 mouse monoclonal; ABCAM) and pNrf2 (anti NRF2 (phosphoS40) antibody (EP1809Y) ab76026 rabbit monoclonal; ABCAM) antibody (1:300 each) was prepared in dilution solution (blocking solution 1:5 diluted). 30 µL/well of Nrf2/pNrf2 antibody mix was added to the cells and incubated over night at 4 °C. After removal of antibody staining solution (1200 rcf, 3 min) cells were washed with DPBS plus 0.1% Triton X. Subsequently, 30 µL/well of secondary antibody mix was added, consisting of IgG anti mouse (donkey anti-mouse IgG (H + L) secondary antibody, Alexa Fluor 488, Invitrogen™, A21202) and IgG anti rabbit (donkey anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 568, Invitrogen™, A10042) antibody diluted 1:500 each in dilution solution. Cells were incubated for 2 h in the dark at room temperature. After two washing steps with DPBS plus 0.1% Triton X two drops of ROTI-Mount FluorCare DAPI (Roth HP20) were added to the cells for staining of nuclei and incubated over night at 4 °C. Fluorescence detection and data analysis was performed with the Cytation 3 cell imaging multimode reader from BioTek Instruments using the software Gen 5.3.3.

For method validation THP-1 monocytes (German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany) were used. Cells were cultured in RPMI 1640 cell culture medium, containing 2 $g \cdot L^{-1}$ glucose, 10% heat inactivated fetal calf serum (FCS) and 45 units$\cdot mL^{-1}$ (1%) penicillin/streptomycin (Thermo Fisher Scientific, Vienna, Austria) at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were incubated for 3 h either with lipopolysaccharides from Escherichia coli (LPS from *E. coli*; 5 ng/mL) or with the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO; 1 µM) and subsequently prepared for immune-fluorescence imaging analysis following the same procedure as used for lymphocytes of the intervention study.

2.5.1. Settings

The Gen 5.3.3 software encompasses tools for the setting of so-called masks, enabling the measurement of translocation events between different cellular compartments. These settings facilitate proper quantification of the fluorescence signal. Fig. 1 illustrates the settings for the measurements of Nrf2 and pNrf2 translocation analysis exemplified by lymphocytes of participants of the intervention study.

The DAPI staining of nuclei served as a first mark identifying the location of nuclei and thus, number of cells within the optical field (mask 1; Fig. 1). The cell number facilitates fluorescence reading per cell. Furthermore, DAPI was used to distinguish lymphocytes from cell debris, which was excluded from the measured fluorescence signal by setting mask 1. By this, the nuclear fluorescence intensity of Nrf2 and phosphorylated Nrf2 (pNrf2) was assessed as the area under the peak of the fluorescence curve. Fluorescence intensity of the whole cell was determined by enlarging the area surrounding the nucleus by 3 µm. This setting, mask 1 + 2 (Fig. 1), allows the quantitation of the Nrf2 and pNrf2 signal in the whole cell. The difference between fluorescence of the whole cell and the nucleus gives the cytosolic fluorescence intensity per cell.

2.5.2. Analysis

The median of fluorescence intensity/cell obtained with the Gen.5.3.3 software of at least 3 optical fields (corresponds to 3 different wells of a 96-well plate) was calculated for Nrf2 and pNrf2 for the nucleus, whole cell and cytosol using Microsoft Excel 2013. The ratio between the signal of the nucleus and the whole cell is considered as R-factor, depicted as median ± SD. The R-factor takes into account variations in fluorescence signal intensity due to suboptimal resolution of cells within the optical field. An increase of the R-factor at visit 2 in comparison to visit 1 would indicate translocation of Nrf2 or pNrf2 into the nucleus of PBLs.

2.6. Transcription analysis of NFE2L2 and KEAP1

Total RNA and genomic DNA was isolated simultaneously from lymphocytes with the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's protocol at the day of blood draw and stored at −80 °C until further need. RNA yield varied quite a bit between the different volunteers, on the one hand potentially due to variations between the different volunteers' concentration of blood lymphocytes and on the other hand due to varying recoveries of PBLs from lymphocyte preparations. Hence, for qRT-PCR analysis always the maximum amount of RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's recommendations. Complementary DNA (cDNA, 20 ng/reaction) was used as template for gene-specific amplification in the PCR reaction comprising QuantiTect SYBR Green Master Mix (Qiagen) and gene-specific primer assays from Qiagen. Following primer assays were used: Nrf2 (NFE2L2): Hs_NFE2L2_1_SG, QT00027384; Keap1 (KEAP1): Hs_KEAP1_1_SG, QT00080220; β-actin (ACTB): Hs_ACTB_1_SG, QT00095431; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Hs_GAPDH_1_SG, QT00079247. Each sample was analyzed in duplicate following the universal PCR protocol: incubation at 95 °C for 15 min, thereafter 40 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec followed by melting curve settings. Quantitative real time PCR was performed with the StepOne Plus system (Applied Biosystems) using the StepOne software for data (C_t-

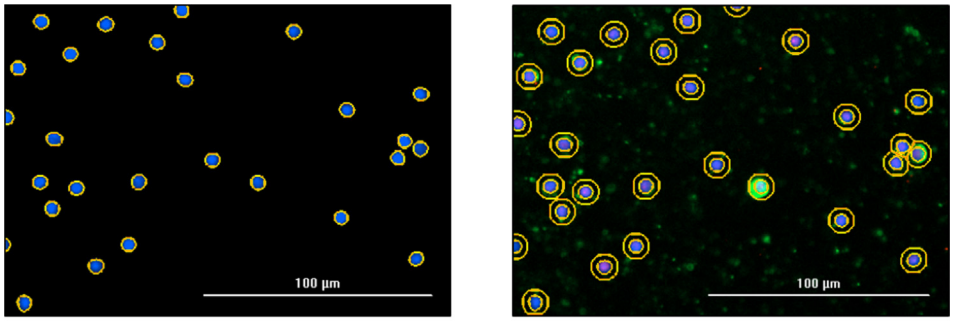


Fig. 1. Mask-settings for quantitation of fluorescence signal for Nrf2 and pNrf2 in human lymphocytes applying Gen 5.3.3 software for immune-fluorescence imaging analysis.

values) collection. Transcript levels of target genes relative to the mean of both control genes ACTB and GAPDH were calculated with Microsoft Excel 2013. Relative quantity of normalized target genes was quantitated according to the $\Delta\Delta C_t$-method, as PCR efficiencies were comparable.

2.7. Statistical analysis

The sample size was calculated for the comparison between coffee and control group with respect to levels of DNA strand breaks in blood cells as the primary endpoint. Normally distributed data were assumed. The significance level was set at 5% and the power of the test was required to be 80%. The expected effect size was derived from the results of Bakuradze et al. leading to an estimated sample size of 39 per group (Bakuradze et al., 2014). Calculating a drop-out rate of about 10% and accounting for possibly higher standard deviations induced by the inclusion of women, 50 subjects per group seemed appropriate. The corresponding calculations were performed with G*Power 3.0.10 software. The participants were assigned to treatment groups by a block randomization procedure.

The software SAS V9.0 was used for computational statistics testing the following statistical hypotheses for confirmation. The hypothesis of no differences between treatment groups was tested against two sided alternatives. Repeated measurements per visit (V) in case of Nrf2 translocation parameters were summarized by the median. Since absolute values for Nrf2 translocation parameters (nucleus, whole cell and cytosol) were considered as not comparable between individual volunteers ratios V2/V1 were calculated. As a consequence, tests for homogeneity of groups at baseline were not applicable for those parameters.

The hypothesis of normally distributed variables was checked by the Shapiro-Wilk test. In case of non-rejection of the normality hypothesis, analysis of covariance (ANCOVA) was performed in order to compare the test and the control group with baseline values as covariates. Ratios V2/V1 were analyzed by *t*-test for independent samples. If the hypothesis of normally distributed data was rejected, Wilcoxoń rank sum test (WRST) was used.

Homogeneity of groups at baseline was checked by *t*-test. In case of rejection of the hypothesis of normally distributed data the WRST was used alternatively. Changes over time per treatment group were evaluated by *t*-tests for paired samples or Wilcoxon signed rank test. When working with ratios V1/V2 the null-hypothesis of no changes over time was formulated as “ratio V1/V2 equals 1”.

Since only two samples were compared, a p-value adjustment for multiple comparisons was not necessary. Analyses were based on both intention to treat (ITT) and per protocol (PP) population to allow for comparison. Data from noncompliant subjects or subjects with missing observations were excluded for evaluation of the PP population. In contrast to PP analysis baseline values were carried forward for ITT analysis.

ITT Population: ITT population consisted of 96 volunteers, 23 men and 25 women allocated to each of the treatment groups (control and coffee). One volunteer of the test group dropped out in the course of intervention. Missing measurements for Visit 2 were replaced by carrying forward the observed values at Visit 1.

PP Population: In 10 samples signs of non-compliance were detected in urine samples, for 8 participants showing higher NMP/creatinine concentration than it should be expected under abstention from coffee consumption (i.e. > 0.2 nmol/µmol, (Lang, Wahl, Stark, & Hofmann, 2011)). In 2 samples of the coffee group no NMP was detectable, suggesting no coffee consumption. Consequently, the samples were excluded and thus data of these volunteers from PP analysis. Finally PP population consisted of 44 volunteers (22 men and 22 women) allocated to control group and 42 volunteers (21 men and 21 women) allocated to the coffee group. Concerning the spontaneous DNA strand breaks some additional values were excluded from PP analysis. At visit 2 the randomly selected control sample (UV-B irradiated PBL) of a sample set (n = 11) signaled a problem in comet assay processing in the course of evaluation. As a consequence, this sample set was eliminated from PP analysis. Regarding the analysis of Nrf2 translocation sample size was reduced as well (n = 66), due to partially highly degraded lymphocytes.

3. Results and discussion

3.1. Effect of coffee consumption on DNA integrity

The impact of dark coffee consumption (coffee C21) on spontaneous (background) DNA strand breaks was assessed with the comet assay in blood samples of healthy male and female volunteers. Study groups were comparable in age, BMI and body weight (Hochkogler et al., 2019). DNA strand breaks detected with the comet assay are one of the most reliable biomarkers to indicate early biological effects (Anderson, Dhawan, & Laubenthal, 2013; A. Collins et al., 2014). Besides “drop-outs” and values of non-compliance of several volunteers of the control (n = 4) and coffee (n = 5) group (Hochkogler et al., 2019), some additional values were excluded from PP analysis (see Section 2.7 statistical analysis).

At the end of the 8 week intervention mean spontaneous DNA strand breaks were 0.09 TI(%) lower (mean delta value) in the coffee drinking group (n_{coffee} = 37) compared to the control group ($n_{control}$ = 38) (Fig. 2). This difference was statistically significant (ANCOVA, p = 0.0262), suggesting a protective effect of the coffee blend with respect to spontaneous DNA damage. The mean change over time of spontaneous DNA strand breaks (−0.06) in the control group was statistically not significant (*t*-test, p = 0.1746). After the “run-in” period, at baseline no significant difference in means of DNA strand breaks was detected between coffee (n_{coffee} = 42) and control group ($n_{control}$ = 44) (*t*-test p = 0.4531). Considering ITT population, median spontaneous DNA strand breaks were 0.10 TI (%) lower (median delta value) in the coffee group (n_{coffee} = 48) compared to the control group ($n_{control}$ = 48) at the end of the intervention period. This difference was statistically significant (WRST, p = 0.0467) reassuring the results obtained by PP analysis. The median change over time in spontaneous DNA strand breaks (0.03) in the control group ($n_{control}$ = 48) was also statistically not significant (WSRT, p = 0.3199). After preconditioning, at baseline no significant difference in medians between coffee (n_{coffee} = 48) and control group ($n_{control}$ = 48) was detected (WRST, p = 0.2545).

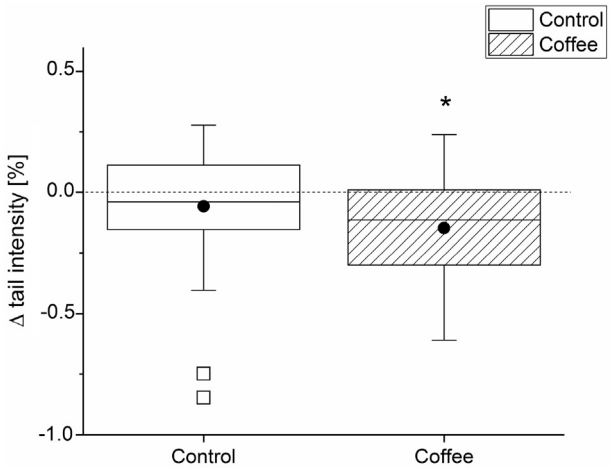


Fig. 2. Absolute changes of spontaneous DNA strand breaks [TI%] in blood cells of water (control) and coffee drinking participants from visit 1 (end of “wash-out” period) to visit 2 (end of intervention), PP population (ANCOVA, * = p = 0.0262).

The reduction of background DNA damage in healthy volunteers by the special blend of pure *Arabica* roast coffee is in accordance with observations of Bakuradze et al. (2011, 2015, 2016, 2010, 2014). It is also supported by results of the intervention study of Hoelzl et al. (2010) showing decreased DNA strand breaks in isolated lymphocytes after the intake of instant coffee rich in chlorogenic acids. The decrease of spontaneous DNA strand breaks by 0.09 TI(%) after 8 weeks consumption of the test coffee fits also the reducing range of the short-term intervention study with repeated coffee intake (Bakuradze et al., 2016). There, over a time period of 8 h an *Arabica* coffee blend was consumed every 2 h (200 mL) resulting in a maximum decrease of DNA strand breaks by 0.11 TI(%). Shaposhnikov and colleagues reported no effect of coffee consumption (up to 5 cups) on background DNA damage in peripheral blood mononuclear cells of healthy volunteers (Shaposhnikov et al., 2018). Despite differences in sample material (isolated blood cells versus whole blood) used in the comet assay and in study design, the most striking difference persists between the study coffees, as can be deduced from well-established roast kinetics for bioactives in coffee (Lang et al., 2013). Shaposhinkov et al. used a rather light-roast coffee blend. In contrast, in the present study a dark-roast coffee blend with the pronounced composition of such a coffee was consumed. Results of two human intervention studies with different designs yet with 4 weeks of dark roast coffee consumption (Bakuradze et al., 2014, 2015) support the protective effect of the similar composite coffee blend used in the present study. However, the potency of the coffee is less pronounced. Spontaneous DNA strand breaks were reduced by 0.09 TI(%) compared to 0.13 TI(%) in the Bakuradze study (Bakuradze et al., 2015). The minor deviation might originate from differences in study design and population (in the present study male and females participated), duration of intervention (8 weeks instead of 4 weeks) and the change in operator and statistical analysis of the comet assay (200 cells were scored instead of 100). Variations in coffee composition, but also differences in environmental conditions such as insolation, season, as well as kinesic behavior (Møller, Loft, Lundby, & NV, 2001) of the volunteers can potentially affect redox-sensitive signaling pathways and thus background DNA integrity.

3.2. Effect of coffee consumption on NFE2L2 and KEAP1 transcription

The question whether in lymphocytes the special coffee blend has an impact on the highly sensitive detector of oxidative stress Nrf2 and its main regulator Keap1 was addressed by transcription analysis using two-step real time PCR. In several studies, a correlation between coffee and Nrf2-regulated detoxifying enzymes has already been described (Boettler, Sommerfeld et al., 2011; Boettler et al., 2012; Vicente, Ishimoto, & Torres, 2014; Volz et al., 2012). Even a moderate up-regulation of Nrf2 itself was reported after coffee consumption (Boettler et al., 2011a). Thus, a potential impact of the study coffee was first assessed at the transcription level focusing on the key players of oxidative stress response.

The results show no statistically significant difference of NFE2L2 mRNA levels between the coffee drinking group and the control group, indicating no impact of coffee on the transcription of the pivotal oxidative stress sensor Nrf2 (Fig. 3A). Transcript levels of KEAP1, the main regulator of Nrf2, were not affected by coffee consumption as well (Fig. 3B).

Based on the analysis of PP population, the difference in median changes of normalized transcript levels of NEF2L2 (visit 2 - visit 1) between treatment groups ($n_{control}$ = 44, n_{coffee} = 42) was 0.03. This difference was not significant (WRST, p = 0.9415). It is important to mention in this context, that the median value of normalized NEF2L2 transcript levels of the control group over time was significantly higher at visit 2 compared to the median value at visit 1 (p = 0.0218, WSRT, Fig. 3C), albeit the groups were homogeneous at baseline (p = 0.4977, WRST). This trend certainly affected the overall result of the NEF2L2 transcription analysis. ITT analysis confirmed the results of the PP

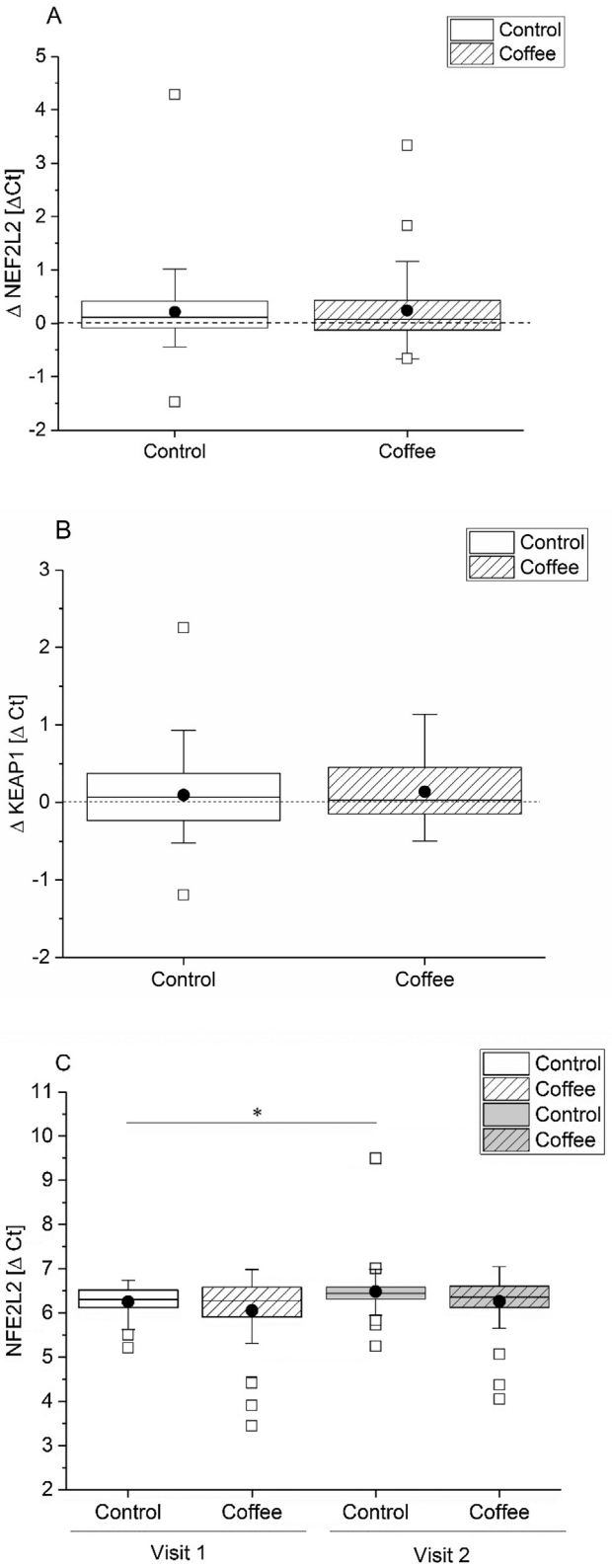


Fig. 3. Modulation of Nrf2 and Keap1 transcription by coffee consumption. (A) Absolute changes of normalized NFE2L2 (Nrf2) transcript levels and (B) KEAP1 transcript levels in human PBLs from visit 1 (end of "wash-out" period) in comparison to visit 2 (end of intervention), PP population. (C) Normalized NEF2L2 transcript levels in human PBLs from control and coffee group at visit 1 and visit 2, PP population (WSRT, * = p = 0.0218).

analysis.

By analyzing the transcription data of NFE2L2 in more detail, several participants could be identified with slightly increased NFE2L2 transcript levels after consumption of coffee (RQ ≥ 1.4; n = 7). These participants might be considered as potential weak responders to coffee. However, it is difficult to assign these candidates clearly to responders or weak responders since no information is available about the NFE2L2 genotypes of the study group. In comparison to the results of Boettler et al. (2012) the induction of NFE2L2 transcription in the present study however is minor and thus could be considered at best as a tendency. Also, the number of potential responders is considerably lower as has been reported by Boettler and colleagues, indicating a potentially minor effect of the present study coffee in this respect.

Based on the analysis of PP population, the difference in median changes of normalized transcript levels of KEAP1 (visit 2 – visit 1) between treatment groups ($n_{control}$ = 44, n_{coffee} = 42) was 0.04 (Fig. 3B). This difference was as well not significant (p = 0.5774, WRST). Median values of the control group did not change significantly from visit 1 to visit 2 (p = 0.4406, WSRT). The groups were homogeneous at baseline with respect to KEAP1 (p = 0.6626, WRST). ITT analysis confirmed the results of the PP analysis.

In summary, no statistically significant effect of the coffee blend was observed with respect to NFE2L2 and KEAP1 mRNA levels in PBLs of the participants. Thus, the transcription of the key regulators of Nrf2 signaling was not affected after 8 weeks of coffee consumption.

3.3. Impact of coffee consumption on Nrf2 translocation in lymphocytes

A missing effect of coffee consumption on NFE2L2 or KEAP1 transcript levels in lymphocytes does not necessarily imply that there is no impact on Nrf2/ARE signaling at all. Enzymes bearing an ARE in their promoter region, such as NQO1, GSTs and even NFE2L2, have been described to be induced by coffee and coffee constituents *in vitro* and *in vivo* (Volz et al., 2012). Ultimately crucial in this context is the translocation of the transcription factor Nrf2 into the nucleus and the binding to the ARE-sequence resulting in the induction of respective enzymes protecting from oxidative stress or toxins. Therefore, we investigated the ability of the study coffee to provoke Nrf2 translocation in blood lymphocytes of the volunteers. The activation of the Nrf2/ARE pathway usually represents a short time event occurring within few hours as has been shown *in vitro* and *in vivo* (Boettler, Volz et al., 2011; Volz et al., 2012). For the 8 weeks long-term intervention with daily coffee consumption, we hypothesized that in case of Nrf2 activation, the coffee drinking population might possess increased nuclear Nrf2 levels in their lymphocytes in comparison to the water control group. However, measuring only the amount of nuclear Nrf2 in lymphocytes of different participants is not a reliable parameter for Nrf2 translocation due to high variations in the amount of Nrf2 protein in lymphocytes of the blood circulation. Thus, the ratio of Nrf2 levels between nucleus and whole cell (R-factor) was chosen to give more reliable evidence for translocation. Since both, Nrf2 and phosphorylated Nrf2, are described to be involved in the induction of Nrf2/ARE dependent gene transcription and it is not really clear which form of Nrf2 is preferably targeted by coffee, we captured both by immune-fluorescence imaging.

In Fig. 4A respecting imaging results of PBLs are shown exemplary from water (control) and a coffee drinking participant of the trial. Assigned to the images are the respective graphically plotted fluorescence intensities of the whole cell, nucleus as well as of the ratio between the fluorescence signal of the nucleus and the whole cell, being defined as R-factor.

The R-factor for Nrf2 and pNrf2 of the water drinking volunteer is in visit 1 and 2 almost identical. Thus, neither Nrf2 nor pNrf2 were affected by water in the PBLs of the participant. In contrast, after 8 weeks (visit 2) the R-factor for pNrf2 of the coffee drinking volunteer is marginally yet significantly increased in comparison to visit 1, indicating translocation of pNrf2 to the nucleus. Whereas the phosphorylated Nrf2 was clearly affected after 8 weeks of coffee consumption, the non-phosphorylated form of Nrf2 was not. The two exemplarily chosen samples also illustrate the broad intra- and inter-individual discrepancies in Nrf2 and pNrf2 content of human PBLs in the blood circulation. The comparison of the results obtained in human PBLs to data obtained from human monocytic leukemia cells THP-1 incubated with well-known activators of Nrf2 translocation such as lipopolysaccharide (LPS) and the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) verify the validity of the method. Limited availability of lymphocytes from participants of the intervention study lead to the use of the THP-1 cells for method validation. Both compounds significantly increased the relative fluorescence intensity of nucleus to whole cell (R-factor) for Nrf2, indicative for translocation into the nucleus (Fig. 4B) whereas translocation of pNrf2 was not affected (Fig. 4C).

3.3.1. Impact of coffee consumption on Nrf2

During the intervention period the median ratio of fluorescence intensity for Nrf2 of nucleus to whole cell increased by 0.03 in the coffee group (n_{coffee} = 36) and by 0.02 in the control ($n_{control}$ = 30) group according to analysis of PP population (Fig. 5A). The difference in changes between the groups was statistically not significant (p = 0.6756, WRST), indicating no translocation of Nrf2 in the coffee drinking group. Fluorescence intensity in the control group ($n_{control}$ = 30) did not significantly change over time (p = 0.0614, WSRT).

During the intervention period, median fluorescence intensity of Nrf2 per whole PBL decreased in the coffee group by 6% and in the control group by 3% (see Fig. 5B). The difference in relative changes between the groups was statistically not significant (p = 0.9846, WRST). A rise in median fluorescence intensity per cell for Nrf2 would have indicated a potential increase in Nrf2 expression, however a rather decrease was observed indicating events associated with a loss of Nrf2 fluorescence signal such as for instance phosphorylation by particular kinases. This is conceivable since according to the provider's information the anti-Nrf2 antibody detects exclusively the non-phosphorylated form of Nrf2. The merged images of Nrf2 and pNrf2 in Fig. 4A clearly support this information since no sign of overlay (yellow color) is visible which would indicate overlapping detection of the two Nrf2 forms. Thus, it can be speculated, that as soon as Nrf2 is phosphorylated in response to coffee a drop in signal intensity for Nrf2 would be the result. In addition, the image analysis data regarding Nrf2 expression in lymphocytes of the participants of the intervention trial underpin the results observed at the transcription level, indicating no increase of NFE2L2 transcripts and thus as expected no increase of Nrf2 protein.

3.3.2. Impact of coffee on phosphorylated Nrf2

The median ratio of fluorescence intensity of nucleus to whole cell of phosphorylated Nrf2 (pNrf2) increased during the intervention period by 0.06 in the coffee group (n_{coffee} = 36) and by 0.05 in the control ($n_{control}$ = 30) group (Fig. 6A). The difference in changes between the groups was statistically not significant (p = 0.5493, WRST), indicating no translocation of phosphorylated Nrf2 in the coffee drinking group in comparison to the control group. Fluorescence intensity in the control group did not significantly change over time (p = 0.0645, WSRT, Fig. 6B).

A more detailed look into the data of the image analysis of pNrf2 however might give some clues about the potential impact of the study coffee, even if no significant translocation of pNrf2 is evident according to Wilcoxon rank sum test. During the intervention period mean fluorescence intensity per nucleus increased in the coffee (n_{coffee} = 36) group by 19% and by 3% in the control ($n_{control}$ = 30) group (Table 2A), indicating potential effects on pathways involved in Nrf2 phosphorylation. Concurrent, the non-phosphorylated form of Nrf2, only detected by the anti-Nrf2 antibody, decreased by 6%, potentially due to a shift towards its phosphorylated form, tending to an increase of

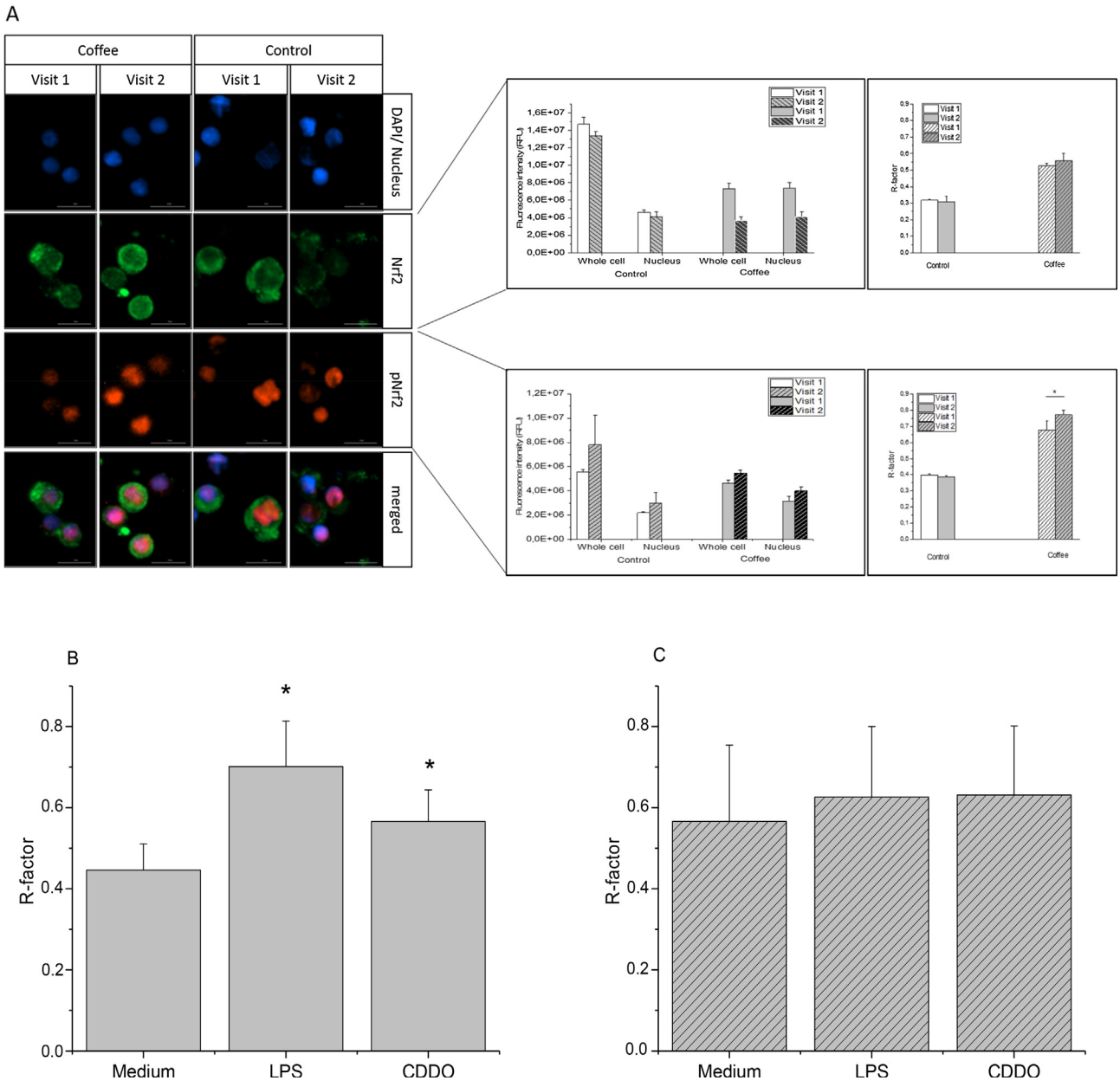
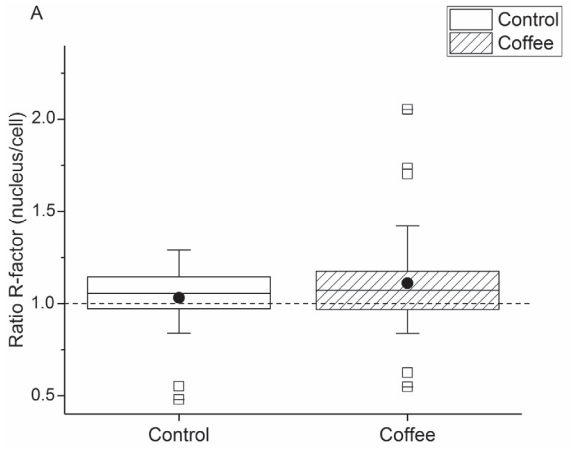


Fig. 4. Immune-fluorescence imaging analysis of Nrf2 and phosphorylated Nrf2. (A) Exemplary images of PBLs from a water (control) and coffee drinking volunteer of the intervention trial and graphical presentation of the fluorescence data from several optical fields. (B) Fluorescence imaging results of Nrf2 and (C) pNrf2 from THP-1 cells exposed to lipopolysaccharide from *E. coli* (LPS) and 2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-oic acid (CDDO), presented as median ± SD of R-factor from at least three independent experiments. The ratio between the fluorescence signal of the nucleus and the whole cell is considered as R-factor. Significances indicated (*) display the significance level as compared to the medium control (one sample *t*-test, * = $p < 0.05$).

median fluorescence of pNrf2 during the intervention. Caffeine for instance is a compound that has been described to impact pathways such as p38MAPK and ERK (Liu, Chen, Cheng, Lin, & Chang, 2013) that are potentially able to phosphorylate Nrf2 as well as to trigger its nuclear translocation (Sun, Huang, & Zhang, 2009). To make matters more complicated and even worse is the fact that Nrf2 undergoes autonomous translocational frequency-modulated oscillations between cytoplasm and nucleus as has been shown in live cell microscopy by Xue et al. (2015). They concluded that frequency modulated translocational oscillations of Nrf2 mediate the ARE-linked cytoprotective transcriptional response rather than a change in total Nrf2 concentration. Thus, based on our data regarding the impact of coffee consumption on Nrf2 signaling, still it cannot be ruled out, that activation of the Nrf2 pathway occurred. Potentially, gene transcription analysis of Nrf2/ARE target genes such as GST1, HO-1, etc. could have shed some light in this problem. However, these analyses were not covered by the approval of the ethics committee. Besides inter-individual variations in basal Nrf2 status and autonomous translocational frequency-modulated oscillations of Nrf2 the limited number of analyzed samples ($n_{coffee} = 36$; $n_{control} = 30$) by fluorescence image analysis needs to be considered. The method was recently established in our lab and planned to be applied explorative in the intervention study. Thus, sample size calculation was not based on fluorescence imaging data but on comet assay data. The results of the present study already show on the one hand that the method is eligible for future studies related to Nrf2 translocation but on the other hand, it is also obvious that the viability of the PBLs is crucial for proper analysis, stipulating a high sample number. Furthermore, since time schemes of intervention and sampling had been oriented on the comet assay and not on Nrf2 translocation, it can only be speculated if such an effect could have been observed more clearly at



B

Nrf2 of whole PBL Ratio visit 2 / visit 1							
Population	**Group**	**N**	**Mean**	**Median**	**SD**	**Min**	**Max**
PP	Coffee	36	1.08	0.94	0.48	0.33	2.23
	Control	30	1.11	0.97	0.57	0.38	3.04

Fig. 5. Modulation of Nrf2 fluorescence signal in PBLs of volunteers participating in the intervention study. (A) Relative changes in nucleus to whole cell ratio from fluorescence intensity of Nrf2 measured in PBLs of volunteers from visit 1 in comparison to visit 2 per treatment group. (B) Descriptive statistics of the PP population. Mean and median fluorescence signal of Nrf2 protein in whole PBLs of the participants are presented as ratio of visit 2 to visit 1 for the control and coffee group. N = number of participants.

another time point of the intervention (Volz et al., 2012).

In summary, the results of fluorescence imaging indicate based on ANCOVA statistics (p = 0.1188) no significant effect of 8 weeks of coffee consumption with respect to Nrf2 translocation.

In line with the implied increase in nuclear pNrf2 after consumption of coffee (see Table 2) is the decrease observed in the cytosol. During the intervention period median fluorescence intensity per cell decreased in the cytosol of the coffee group by 14% and in the control group by 6% (Table 2). The difference in relative changes between the groups however was statistically not significant (p = 0.9640, WRST).

Considering the whole PBL, pNrf2 was still increased in the coffee drinking group. During the intervention period median fluorescence intensity per cell increased in the coffee group by 2% and decreased in the control group by 4% (Table 2), indicating a potential activation of cell signaling pathways resulting in Nrf2 phosphorylation. The difference in relative changes between the groups however was statistically not significant (p = 0.2491, WRST) for reasons mentioned above.

4. Concluding remarks

Chronical consumption (8 weeks) of a pure *Arabica* dark-roast coffee blend (*Coffea arabica* L., coffee C21) significantly reduced spontaneous DNA strand breaks in blood cells of healthy volunteers, pointing to a DNA protective effect. The Nrf2 pathway, one of the protective cellular systems against oxidative stress, appears to be affected yet, at least at this time-point of intervention, shows no significance from the statistical point of view. Considering, that the statistical sample size of the newly established fluorescence imaging method might have been not sufficient, a potential role of Nrf2 signaling in the response to coffee consumption cannot be ruled out entirely.

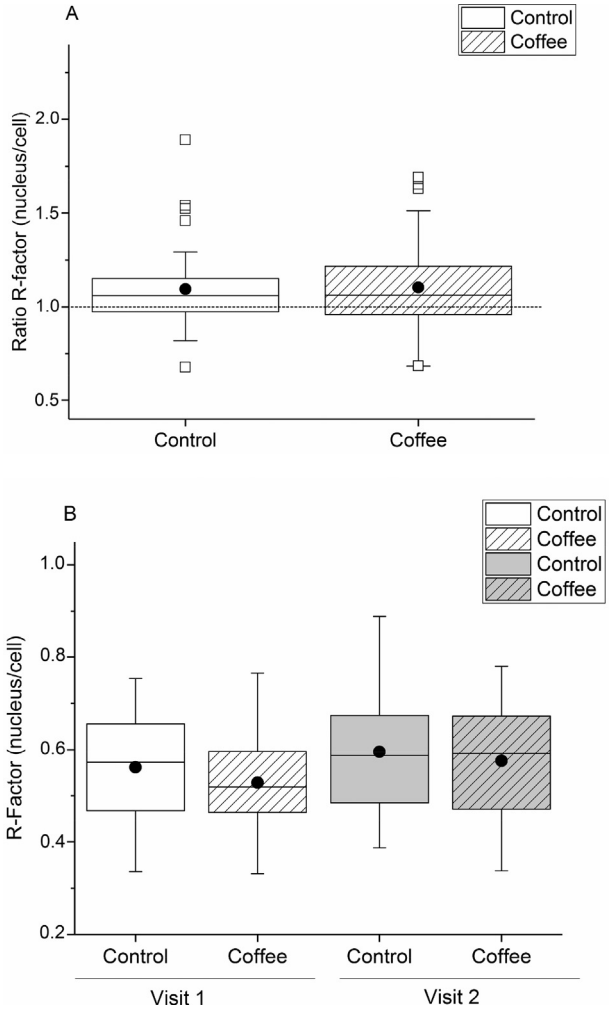


Fig. 6. Modulation of phosphorylated Nrf2 (pNrf2) fluorescence signal in PBLs of volunteers participating in the intervention study. (A) Relative changes in nucleus to whole cell ratio (R-factor) from fluorescence intensity of pNrf2 measured in PBLs of volunteers from visit 1 in comparison to visit 2 per treatment group and (B) Nucleus to whole cell ratio (R-factor) from fluorescence intensity of pNrf2 presented by visit and group.

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Conflict of interest

D. Schipp is a self-employed statistician, who has been retained and financed by Tchibo GmbH for this, and other projects as well. The other authors have declared that no conflicts of interest do exist.

Table 2
Descriptive statistics of data obtained with immune-fluorescence imaging of PBLs from blood samples of the participants of the intervention trial. Presented is the statistical analysis according to PP population for the ratio between visit 2 and visit 1 of the fluorescence signal of pNrf2 in different cell compartments (nucleus; cytosol; whole PBL) for the coffee and water-control group. The pNrf2 signal in the cytosol of PBLs is calculated from the difference of fluorescence signal between whole cell and nucleus, ratio of visit 2 to visit 1. The pNrf2 signal in nucleus and whole PBLs is calculated from the fluorescence signal/nucleus or cell respectively, ratio of visit 2 to visit 1. PP = per protocol N = number of participants, SD = standard deviation.

Ratio visit 2 / visit 1							
Nucleus	**Group**	**N**	**Mean**	**Median**	**SD**	**Min**	**Max**
PP	Coffee	36	1.19	1.09	0.45	0.31	2.29
	Control	30	1.03	1.01	0.31	0.55	1.75
Cytosol	**Group**	**N**	**Mean**	**Median**	**SD**	**Min**	**Max**
PP	Coffee	36	1.04	0.86	0.59	0.41	3.08
	Control	30	0.91	0.94	0.34	0.08	1.61
Whole PBL	**Group**	**N**	**Mean**	**Median**	**SD**	**Min**	**Max**
PP	Coffee	36	1.11	1.02	0.47	0.42	2.33
	Control	30	0.96	0.96	0.28	0.36	1.73

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