Antioxidative response to coffee – depending on human Nrf2 genotype?

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Abstract

In a human intervention trial, a coffee, combining nature green coffee bean constituents and dark roast products was studied towards its potential to activate the Nrf2/ARE-pathway in PBLs. The study coffee was identified as a strong inducer of Nrf2 and downstream GST1A1 and UGT1A1 gene transcription. However, the response of the participants was found to depend on the respective genotype. The -651 SNP in the Nrf2 gene as well as the heterozygote 6/7 sequence in the UGT1A1 gene significantly down-regulated the susceptibility to respond to coffee, proposing the existing genotype to be critical for the response to the coffee.

Keywords:

Reactive oxygen species, antioxidant, coffee, Nrf genotype, GST1A1 genotype, UGT1A1 genotype, genetic variations, gene expression

Phase II enzymes play a critical role in converting reactive electrophiles or xenobiotics into less toxic products and seem to be crucial for cancer prevention. Thus, a deficiency in phase II enzyme activity has been associated with an increased risk of colon cancer [1, 2]. The expression of many phase II genes is regulated via the activation of antioxidant response elements (ARE) which are located in the 5'-flanking region of their respective promoters. Activation by ROS or upstream protein kinases induce the translocation of the transcription factor Nrf2 (Nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2 from the cytoplasm into the nucleus, its binding to ARE and the onset of the transcription of phase II enzymes such as glutathione *S*-transferases (GST), UDP-glucuronyl transferases (UGT) or γ-glutamyl cysteine ligase (γGCL) [3-12].

Recently, we identified the typical dark roast coffee constituent *N*-methylpyridinium (NMP) as well as the green bean constituent n-chlorogenic acid (CGA) as potent activators of the Nrf2/ARE pathway [13].

Four weeks consumption of a coffee blend combining both, typical dark roast characteristics as NMP with considerable amounts of green bean constituents significantly increased mean Nrf2 gene transcripts in peripheral blood lymphocytes (PBLs) of the participants of a human intervention trial [14, 15]. Yet, large differences in the Nrf2 activation ability were detected amongst the individuals, which could not be explained by the differences in the general health status or in the nutritional behavior of the participants.

It has previously been reported that genetic variations in certain antioxidative genes critically determine their capability to be activated by bioactive food compounds [16]. Biochemical studies have associated allelic variations in the Nrf2 promoter region with differences in the ability to increase antioxidative phase II gene transcription [17]. To date, 9 Single Nucleotide Polymorphisms (SNP) have been identified in the Nrf2 gene [17-19]. Of special relevance seems to be the -617C/A polymorphism and the -651G/A SNP, which are located in the promoter region of the gene. Both SNPs were found to reduce the transcriptional activity of Nrf2, reflected by extenuated binding of Nrf2 to the ARE, resulting in decreased Nrf2-dependent gene transcription [17]. Genotyping of a subset of the study collective of HSII hinted at a connection between the respective Nrf2 genotype and the response to coffee [15].

Based on these findings aim of the current intervention trial was (a) to test the Nrf2-activation potential of the study coffee in a larger cohort and (b) to comprise whether

the individual differences in the response to the study coffee is associated with the presence of different genotypes of Nrf2, GST1A1 and UGT1A1 genes.

Results

Nrf2 genotype and gene transcription

A 424 bp polymorphic region of the Nrf2 promotor was sequenced in all participants of the intervention trial to detect the -617C/A, the -653A/G and the -651G/A polymorphisms which have already been discussed to be functionally relevant for the transcriptional activity of the Nrf2 gene [17].

The -617C/A SNP was present in 10/49 (20 %) of the participants (Tab. 1). The presence of the -651G/A SNP was observed in 15/49 individuals (31 %) and 31/49 (63 %) of the participants possessed a SNP at position -653A/G. Changes in Nrf2 gene transcription were assessed at the different blood collection points (CPs) in the course of the study. A potent induction of Nrf2 gene transcription after four weeks consumption of the study coffee (BC3) in comparison to both, wash out (BC2) as well as normal diet (BC1) was detected (Fig.1A). Thus, 65 % (32/49) of all participants displayed a ≥1.5 induction of gene transcription after coffee consumption.

On closer examination of the genotype-dependent magnitude of the Nrf2 activation, -651 SNP carriers displayed a weaker Nrf2 activation after coffee consumption (BC3) than carriers of the WT or any other SNP (Fig. 1B). Comparing the sensitivity of the response between all occurring genotypes displayed that individuals carrying the -617 and the -651 SNP concomitantly possessed the lowest potential to activate Nrf2 gene transcription (data not shown).

GST1A1 genotype and gene transcription

An already know polymorphism in the GST1A1 gene which consists of two alleles, GST1A1∗A and GST1A1∗B was monitored [20]. The two genotypes differ in three linked base changes in the 5'-regulatory region of the gene (a T/G change at −567, a C/T change at −69 and G/A change at −52). Presence of C/T base change at position -69 (GST1A1*B) gave rise to a EAR1 restriction enzyme cutting site, resulting in two fragments of length 380 bp and 100 bp whereas GST1A1*A homozygotes show only one band at 480 bp (data not shown).

From the participants of the intervention trial, 24/48 (50 %) were found to carry an A/A genotype, 18/48 (38 %) the heterozygote A/B genotype and 6/48 (12 %) the more rarely homozygote B/B genotype (Tab. 1). To investigate whether four weeks intervention with the study coffee modulates GST1A1 transcription levels, as an

important member of the antioxidative GST enzyme family, changes in gene transcription were determined during the course of the trial. GST1A1 gene transcription was potently elevated in the mean of the participants after four weeks consumption of coffee (BC3) in comparison to wash out (BC2, Fig. 2A). Separation of the participants with respect to their response to coffee resulted in 54 % of the participants displaying ≥1.5 induction of GST1A1 gene transcription after coffee consumption. Furthermore, this group of coffee responders displayed a higher induction of Nrf2 gene transcription following their individual normal diet (BC1).

Subsequently, the GST1A1 genotype was correlated to the changes in GST1A1 gene transcription during the course of the trial. No significant difference in the magnitude of GST1A1 activation was apparent in relation to the GST1A1 genotype. However, individuals possessing the BB homozygote genotype seemed to be slightly more susceptible to coffee consumption (data not shown).

The GST1A1 gene carries an ARE sequence in its promotor region, hence it transcription is activated by Nrf2. Transcriptional activity of Nrf2, and thus the ability to activate ARE-dependent genes seems to depend on the Nrf2 genotype of the individual. An Nrf2 genotype-dependent magnitude of GST1A1 activation was detected for the study collective. Participants carrying the -651SNP, accounting to 31 % of the participants showed no increase in GST1A1 gene transcription after coffee consumption (Fig. 2B). Thus, the Nrf2 genotype seems to be critical for GST1A1 response.

UGT1A1 represents a further important phase II gene in cellular detoxification processes. The activation of UGT1A1 gene transcription in mice by coffee has already been reported [21]. Thus, studies on changes of UGT1A1 gene transcription in the participants of the intervention trial were included. UGT1A1 possesses a known variation in its promotor sequence [22]. Moreover, the presence of such an additional TA repeat in the TATA region of the UGT1A1 promoter (7 TA repeats; UGT1A1*28) markedly decreases UGT1A1 expression and leads to a reduced sensitivity of the gene [22, 23]. Most participants of the present intervention trial were found to possess either a homozygote $[TA]_{6/6}$-repeat sequence (wild type, 21/46 individuals (46 %)) or were heterozygote ($[TA]_{6/7}$ (19/46 individuals (41 %)), whereas only 13 % (6/46) of the individuals could be identified as homozygote $[TA]_{7/7}$-repeat sequence carriers (Tab. 1).

A strong variation in UGT1A1 gene transcription levels amongst the participants was

detected at the beginning of the trial (BC1, Fig. 3A), whereas the response to coffee seemed to be more distinct (BC3). In average an induction of UGT1A1 gene transcription was observed after four weeks intervention with the study coffee. Furthermore, the absence of the coffee (second wash out period, BC4) resulted in a down-regulation to base level, emphasizing coffee to be crucial for the strong induction at BC3. By separation of the participants into groups of responders (BC3 ≥1.5) and non-responders (BC3 <1.5), 63 % of the individuals could be identified as coffee responders, not only supporting former findings, that coffee activates UGT1A1 gene transcription but even showing this in humans (Fig. 3A).

The changes in UGT1A1 transcription during the trial were correlated to the UGT1A1 genotype with special emphasis to the response to the coffee intervention period, demonstrating a significant correlation between a reduced response to coffee and the presence of the 6/7 heterozygote genotype (Fig. 3B). Individuals carrying either a 6/6 homozygote or a 7/7 heterozygote genotype responded equally sensitive to coffee.

Correlating the response in UGT1A1 gene transcription to the Nrf2 genotype indicated the -651 Nrf2 genotype to be less accessible to coffee-mediated onset of gene transcription. Individuals, carrying the -651 Nrf2 genotype showed no increase in UGT1A1 gene transcription after coffee consumption (BC3) in comparison to wash out (BC2) whereas individuals, carrying the WT, -617SNP or the -653SNP displayed a potent onset of UGT1A1 gene transcription after coffee consumption (Fig. 3C).

Linking the presence of the less sensitive responding 6/7 heterozygote UGT1A1 genotype to the less sensitive -651 Nrf2 genotype displayed that 63 % (5/8) of individuals carrying both, the 6/7 UGT1A1 and the -651 Nrf2 genotype at the same time belonged to the group of coffee non-responders hinting at a strong correlation between the genetic background of the individuals and their response to coffee.

Discussion

The study coffee was identified as potent inducer of the antioxidative and chemopreventive Nrf2/ARE pathway in humans, as exemplified by a significant increase in Nrf2, GST1A1 and UGT1A1 gene transcription after four weeks consumption in the mean of the 51 participants, but yet with large differences in the individual response to the study coffee (Fig. 1A, 2A, 3A). Having a closer look on the particular response of the participants revealed for each gene a larger group of responders (Nrf2 65 % (32/49); GST1A1 54 % (26/48) and UGT1A1 63 % (29/46))

showing ≥1.5 fold increase in gene transcription after coffee intervention (BC3) in comparison to the previous wash out period (BC2) as well as a smaller group of non-responders (Nrf2 35 % (17/49); GST1A1 46 % (22/48) and UGT1A1 37 % (17/46)) with <1.5 fold increase in gene transcription after coffee consumption. However, these variations could neither be explained by differences in the uptake of any antioxidative food/ food stuff despite coffee or gender, ethnicity or age. To elucidate, whether the presence of a particular genotype might influence the response to coffee, regions within the Nrf2, GST1A1 and UGT1A1 gene, carrying know polymorphisms already associated to affect the transcriptional activity of the genes were determined [15, 17, 20, 22, 23].

The ratio of most occurring Nrf2 SNPs matches available literature data (Table 1). The -617C/A SNP was present in 10/49 (20 %) of the participants. The frequency of the -651G/A SNP was observed in 15/49 individuals (31 %) and 31/49 (63 %) of the participants displayed a SNP at position -653A/G. Thus, the study collective reflected the highest presence of the -653 SNP in the Nrf2 promotor. This is in line with previously published data [15], however higher than data published by Marzec et al. (2007). Taking into account that the study collective of Marzec et al. (2007) comprised 20 individuals, numbers of the present study (51 individuals) and previously published data (18 individuals) can be stated as more powerful. Thus, a presence of the -653 SNP of 56-63 % in Caucasians seems to be likely.

Moreover, a polymorphism in the GST1A1 gene which consists of the two alleles GST1A1∗A and GST1A1∗B was examined [21]. As given in Table 1, 24/48 (50 %) of the participants were identified as A/A carriers, 18/48 (38 %) possessed the heterozygote A/B genotype and 6/48 (12 %) were B/B carriers. The frequency of the different GST1A1 genotypes is comparable to former findings in Caucasians [20] indicating that the study collective seems to represent an average population profile.

The UGT1A1 gene possesses the known variation UGT1A1*28, which varies among ethnicities, being highest in those of African (43 %) or European (54 %) descent and lowest in those of Asian (16 %) descent [23].The study collective displayed a ratio within the expected range (Table 1). Hence 21/46 individuals (46 %) possessed the wild type sequence ($[TA]_{6/6}$-repeat) and 19/46 participants (41 %) a heterozygote genotype ($[TA]_{6/7}$-repeat). 6/46 individuals (13 %) carried a homozygote $[TA]_{7/7}$-repeat sequence. However, the low presence of this genotype is also supported by literature data [23].

Linking the changes in gene transcription of Nrf2, GST1A1 and UGT1A1 during the course of the two month trial to the respective genotypes displayed several associations. Apparently a genotype-dependent response to coffee was observed for the -651 SNP of Nrf2. Individuals, carrying this genotype showed a considerably lower magnitude of induction in Nrf2 gene transcription during the coffee intervention period (BC3) than participants carrying the WT sequence, a -617 or -653 SNP (Fig. 1B).

UGT1A1 gene transcription was significantly up-regulated in most (63 %) of the participants of the present intervention trial. The UGT1A1 gene also reflected the strongest variation in the response to the study coffee in association to the present genotype. Both, the magnitude of the response, as well as the frequency of induced UGT1A1 gene transcription after coffee consumption was significantly decreased in carriers possessing a heterozygote 6/7 sequence in the determined promotor region. 18/51 (35 %) of the individuals carried this genetic modification and most of this individuals failed to show an increased UGT1A1 gene transcription after coffee consumption (Fig.3B).

Different authors reported a reduced Nrf2 transcriptional activity in individuals carrying genetic variations in the Nrf2 promotor region [17, 18], with effects on downstream ARE-dependent gene transcription. In the present study, individuals carrying the SNP at -651 in the Nrf2 gene reflected a significantly reduced response to coffee, apparent in a significantly lower induction of Nrf2-dependent GST1A1 and UGT1A1 gene transcription (Fig. 2B, 3C). These findings are supported by Marzec et al. (2007) who detected a reduced transcriptional activity of Nrf2, reflected by an extenuated binding of Nrf2 to the ARE (EpRE) in the presence of the -651 SNP. Furthermore, individuals carrying this genetic variation seem to suffer more frequently from acute lung injury (ALI), a disease, related to a reduced antioxidative cell defence [17]. However, his findings explained the influence of the genotype on general/ physiological gene transcription, whereas the effects on the transcriptional activation after food intervention were investigated, here.

Taken together, the study coffee, combining potent Nrf2 inducers from green coffee beans with typical high roasted constituents could be identified as a potent inducer of antioxidative cell protection in the mean of the participants of the two month coffee

intervention trial. Differences in the response to the coffee within the study collective, resulting in a group of responders and non-responders for the transcription of Nrf2, GST1A1, UGT1A1 in peripheral blood lymphocytes could be associated, an effect, which seems to occur in most human intervention trial. The occurrence of the -651 SNP in the Nrf2 gene as well as the presence of a heterozygote 6/7 sequence in the downstream Nrf2-dependent phase II gene UGT1A1 potently down-regulated the susceptibility to respond to coffee with an increase in antioxidative gene transcription. We therefore suggest the genotype to be considered as important background criteria to evaluate the outcome in human intervention trials, targeting Nrf2-dependent gene response and thus, to estimate the chemopreventive potency of bioactive food/ food constituents.

Methods

Coffee brew

The study coffee brew consisted of a special roasted and blended Arabica coffee, characterized by a high concentration of both, green and roasted bean constituents, especially n-chlorogenic acid (252 mg/L) and NMP (76 mg/L). The caffeine level (720 mg/L) was in an average range of conventional coffee brews. The ground coffee was delivered in vacuum bags, each containing a filter pad of 12.5 g study coffee. Immediately before consumption, the coffee brew was freshly prepared by a Senseo coffee maker (Philips) by putting two pads into the large strainer and pushing the “two-cup” button. Each individual consumed 250 mL (three times a day).

Subjects

The study was approved by the Griffith University Human Research Ethics Committee (EC00162). The final test population was comprised of 25 male and 26 female (total n = 51) healthy non-smoking (age 18-61, BMI 19-38) Caucasians. Originally, 57 individuals were recruited, however 6 individuals dropped out because of several reasons (change in living situation: moving away, new job situation and incompatibilities (gastric symptoms or sleeplessness after coffee consumption). All participants had to be regular coffee drinkers (2-6 cups of coffee/d). Participants were asked to maintain their usual dietary habits for the duration of the study, except for the intake of coffee, caffeinated products, dietary supplements and foods rich in

polyphenols. All volunteers were informed of the objectives of the study and consent received for their participation. Exclusion criteria were smoking and use of medication. Furthermore, competitive athlete could not apply. The 8 week intervention trial was designed as follows: week 1+2, 1st wash out; week 3-6, coffee consumption and week 7+8, 2nd wash out. The participants consumed 750 mL of freshly brewed coffee (with/without sugar, addition of milk up to 50 mL) in three equal portions (morning, noontime, afternoon). In the wash out periods, the coffee brew was replaced by equal volumes of water. Nutritional reports during a 7-day periods were completed by the participants in the last week of each study period. Urine/blood sampling was performed at the beginning of the study and at the last day of each study period in the morning, after a fasting period of at least 6 hours in advance.

Genomic DNA isolation

For genomic DNA (gDNA) isolation, the QIAamp DNA Mini kit (QIAGEN) was applied and gDNA was purified as per the manufacturer's instructions. Briefly, 20 µl of proteinase K were pipetted into the bottom of a 1.5 mL tube and 200 µL of whole blood were added. Thereafter, 200 µL of Buffer AL were added and vortexed for 15 s. After incubation at 56 °C for 10 min and brief centrifugation, 200 µL of ethanol (96 %) were added and again mixed by vortexing for 15 s. The mixture was then applied to a spin column and centrifuged at 6000 x for 1 min. Subsequently, the spin column was placed in a clean 2 ml collection tube (the filtrate was discarded). 500 µL of Buffer AW1 were added onto the spin column and centrifuged at 6000 x g for 1 min. Thereafter, the spin column was placed in a clean 2 ml collection tube (the filtrate was discarded), 500 µL Buffer AW2 were added and centrifuged at full speed for 3 min. Subsequently, the spin column was placed in a clean 1.5 mL tube and 200 µL H2O dest. was added. After 1 min incubation at RT a centrifugation step at 6000 x g for 1 min, the final concentration of DNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware).

Sequencing

SNPs rs35652124, rs6706649 and rs6721961:

To obtain the 424 bp polymorphic region of the NFE2L2 gene, 80 ng of gDNA was amplified with 4 µL of GoTaq® Flexi buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each NFE2L2 primer (forward primer 5'–GACCACTCTCCGACCTAAAGG–3' and reverse primer 5'–CGAGATAAAGAGTTGTTTGCGAA-3') and 0.2 µL GoTaq® to a total reaction volume of 20 µL. Amplification was performed on a Veriti™ 96-well

Thermal Cycler (Applied Biosystems) with an initial step of 94 °C for 10 min, followed by 30 cycles of 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 45 s followed by a final extension of 72 °C for 7 min. Following amplification, 5 µL of PCR product was electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80 V. PCR products were purified with the ExoSAP-IT® PCR cleanup kit (Affymetrix/USB) according to the manufacturer's instructions. Briefly, 5 µL of PCR product, 1 µL of Exo SAP-IT® and 4 µL dH_2O were carefully mixed prior to incubation at 37 °C for 15min, followed by incubations at 80 °C for 15 min and 4 °C for 3 min. DNA concentration was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware) and diluted to a concentration of 20 ng/µL DNA. For the sequencing reaction 1 µL of this DNA was added to 5 µL of BigDye Terminator v3 (BDT v3.1, Applied Biosystems), 1.3 µL of each NFE2L2 primer (one forward and one reverse reaction), 3.0 µL of 5 x BDT v3.1 Sequencing buffer to a final volume of 20uL. Amplification cycles were as follows: 96 °C for 1 min, followed by 30 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min followed by 4 °C for 5 min, 10 °C for 5 min and 4 °C for 2min. The sequencing reaction product was transferred to a 1.5 ml eppendorf tube and ethanol precipitated by adding 2 µL of iced cold 3M sodium acetate (pH 5.2) and 2 µL of 125 mM EDTA (pH 8.0). After adding the sequencing reaction product the sample was vortexed briefly, centrifuged at 10,000 g for 5 minutes, followed by addition of 50 µL of 100% ethanol followed by repeated vortexing and re-spinning the samples at 10,000 g for 5 minutes. Samples were then incubated for 15 min at RT. The product was then precipitated by centrifugation at 10,000 x g followed by incubation at 4 °C for 20 min. The supernatant was removed and the pellet rinsed in 70% ethanol, briefly vortexed and centrifuged again at 10,000 x g at 4 °C for 5 min. The pellet was completely dried using a DNA Speed Vac® (Savant) on high drying mode for 5 min. Samples were then resuspended in 15 µL dH_2O. The purified products were then either stored at -20 °C or directly added into a Micro Amp™ optical 96 well reaction plate (Applied Biosystems) to be analyzed on a 3130 genetic analyzer (Applied Biosystems).

GST1A1 genotyping

GST1A1 genotyping was carried out with Restriction Fragment Length Polymorphism (RFLP). First, PCR amplification was done. Gene-specific primers (forward primer 5'-TGTTGATTGTTTGCCTGAAATT-3'; reverse primer 5'-GTTAACGCTGTCACCGTCCT-3') 'were used to generate a 480 bp PCR product

spanning the site containing the polymorphism. For the amplification of GSTA1 40 ng of gDNA was amplified with 4 µL of GoTag® Flexi buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 µM of each GST1A1 primer and 1 U Taq polymerase was added to a total reaction volume of 20 µL. Amplification was performed on a VeritiTM 96-well Thermal Cycler (Applied Biosystems) with an initial denaturation of 94 °C for 10 min, followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s, followed by a final extension of 72 °C for 7 min. Following amplification, PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80 V. A no template control was included to detect possible contamination issues. This PCR product was then digested with EAR1 (New England Biolabs, Beverly, Massachusetts, USA), which recognizes the base change C/T at promotor position -69 in the hGSTA1∗B allele. The digestion was performed on the PCR reaction products in a 20 µl reaction using 4 U of restriction enzyme Ear1, 2 µL of 10X Buffer and 18 µl PCR product for 5 h at 37◦ C. No template control was included. The digested products were resolved in a 4 % ultrapure agarose gel post-stained with 20 µL ethidium bromide for 10 min. The GST1A1*A genotype produced only a band at 480 bp. A heterozygote GST1A1*A/B resulted in three bands at 480, 380 and 100 bp and the GST1A1*B genotype in two bands at 380 and 100 bp.

UGT1A1 genotyping by microsatellite fragment analysis

First, PCR was performed using one unlabelled and one, at the 5'-end 6-FAM labelled primers (forward primer: 5’-156-FAM/AAGTGAACTCCCTGCTACCTT-3’, reverse primer 5’-CCACTGGGATCAACAGTATCT-3’) resulting in a 253 bp PCR product, flanking the polymorphic TA locus in the promoter region. Briefly, 80 ng of gDNA was amplified with 4 µL of GoTag® Flexi buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 µM of each UGT1A1 primer. 1 U Taq polymerase was added to receive a total reaction volume of 20 µL. Amplification was performed on a VeritiTM 96-well Thermal Cycler (Applied Biosystems) with an initial denaturation of 94 °C for 15 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s, followed by a final extension of 72 °C for 7 min. Following amplification, PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80 V. A no template control was included to detect possible contamination issues. 0.5 µL of PCR product were administered to 0.25 µL 1:10 diluted GeneScan™ 500 LIZ Size Standard (Applied Biosystems) and 9.25 µL of HiDi formamide (Applied Biosystems). Samples were diluted 1:5, subjected to sequencing

on the 3130 genetic analyzer (Applied Biosystems) for $(TA)_n$, and scored via Gene Mapper v4.0 software (Applied Biosystems). Control DNAs from previously sequenced individuals known to have a 6/6, 6/7, or 7/7 genotype were included in the PCR analysis. The amplified product yielded a 93-or a 95-base pair fragment, which corresponded to $(TA)_6$ and $(TA)_7$, respectively.

Gene transcription by Q-PCR

At each of the four different blood collection (BC) points, marking the beginning and the end of the three different periods of the intervention trial venous blood samples were collected in EDTA-tubes and stored at room temperature until the individual sampling period was completed. Thereafter, isolation of human peripheral blood lymphocytes (PBL), RNA extraction and Q-PCR were performed as previously reported [15]. Total RNA was extracted from isolated PBLs following the manufactures handbook of the RNeasy®Mini Kit (QIAGEN, Hilden, Germany). Following this, 2 µg RNA was reverse-transcribed using Oligo-dT primers and the Omniscript® Reverse Transcription Kit (QIAGEN, Hilden, Germany). cDNA obtained from the RT reaction (amount corresponding to 2 µg of total RNA) was subjected to Q-PCR using QuantiTect SYBR® Green PCR (QIAGEN, Hilden, Germany). The primer assays used for the NF2L2 gene was: Hs_NFE2L2_1_SG, QT00027384. ß-Actin: Hs_ACTB_1_SG, QT00095431 GST1A1: Hs_GST1A1_1_SG, QT00060739. GSTT1: Hs_GSTT1_2_SG, QT010751638. UGT1A1: Hs_UGT1A1_1_SG, QT00020860 (QIAGEN, Hilden, Germany). Primer concentrations and Q-PCR reaction parameters were according to manufacturer's guidelines in QuantiTect SYBR® Green PCR Handbook 11/2005 (QIAGEN, Hilden, Germany). Each sample was determined in duplicate. A -RT control was included for all assays. The fold changes in expression of the target gene relative to the internal control gene (ß-actin) was analyzed using Bio-Opticon Software and the C_T data was imported into Microsoft Excel 03. Data of all assays was analyzed by the $2^{-\Delta\Delta C_T}$ method.

Figures

Figure 1A

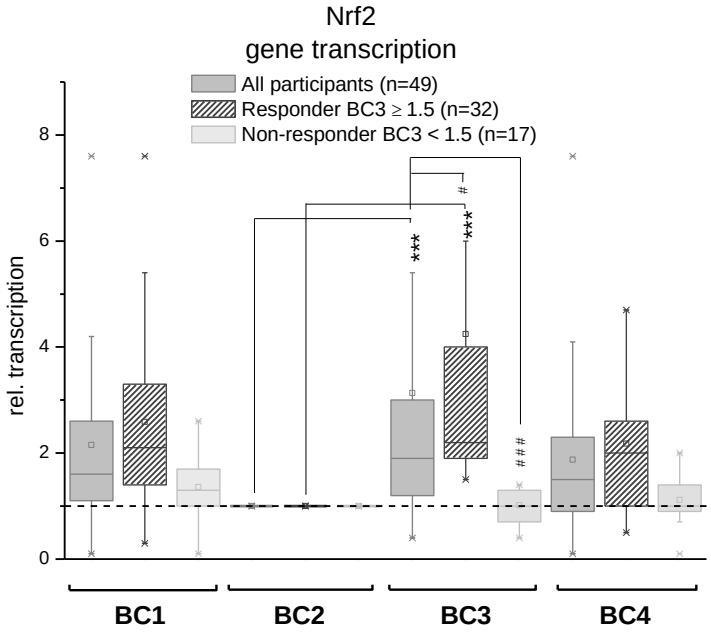

Figure 1B

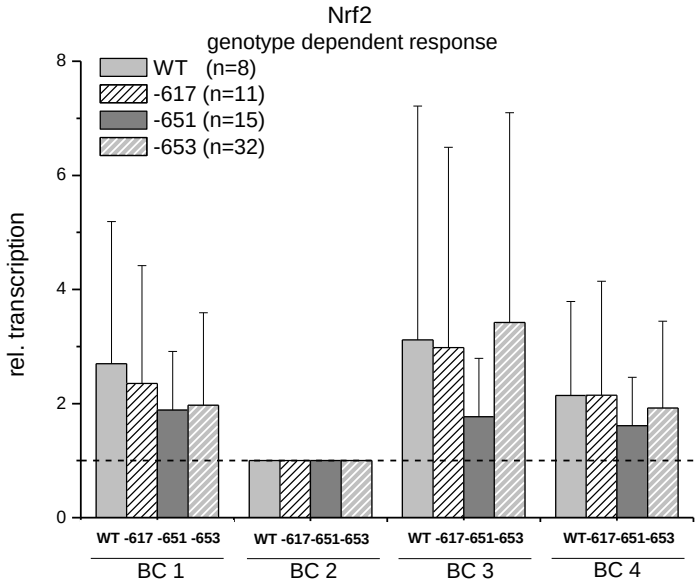

Figure 2A

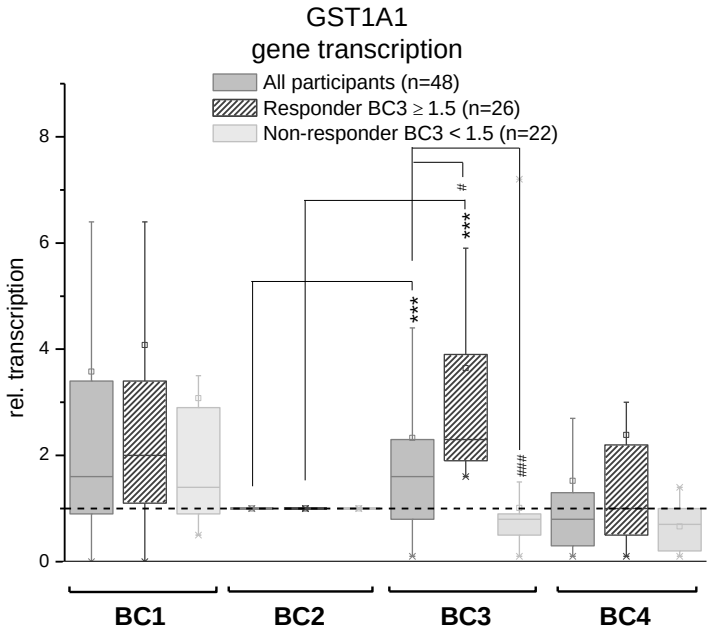

Figure 2B

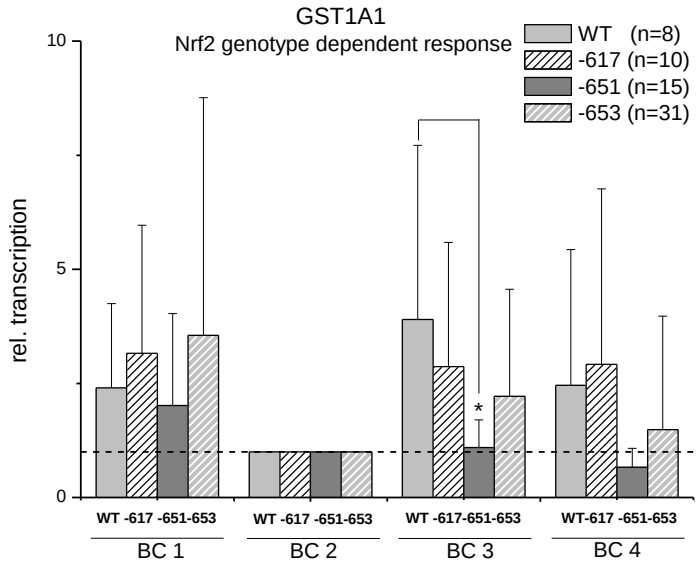

Figure 3A

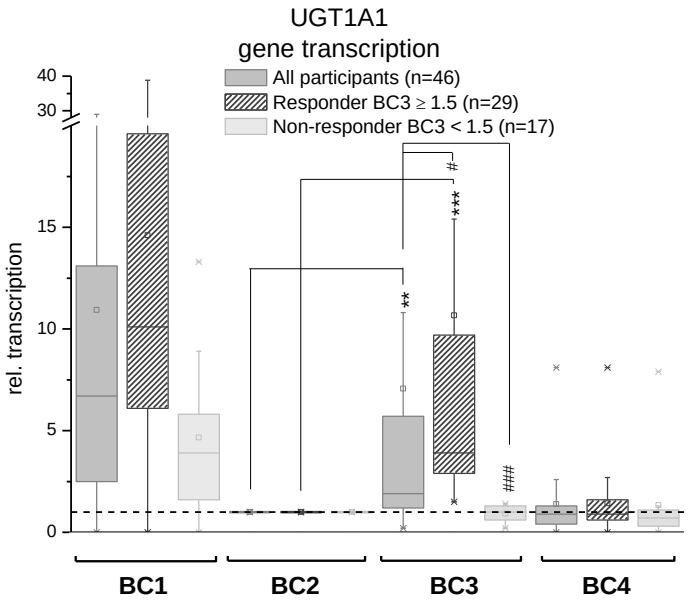

Figure 3B

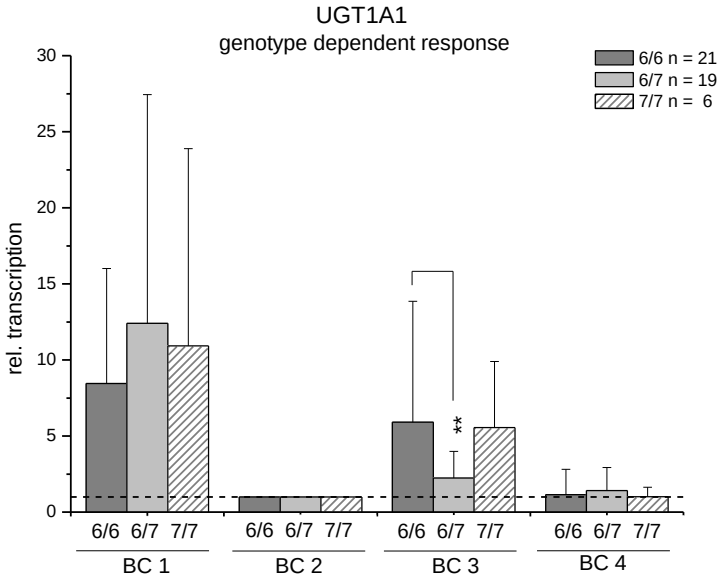

Figure 3C

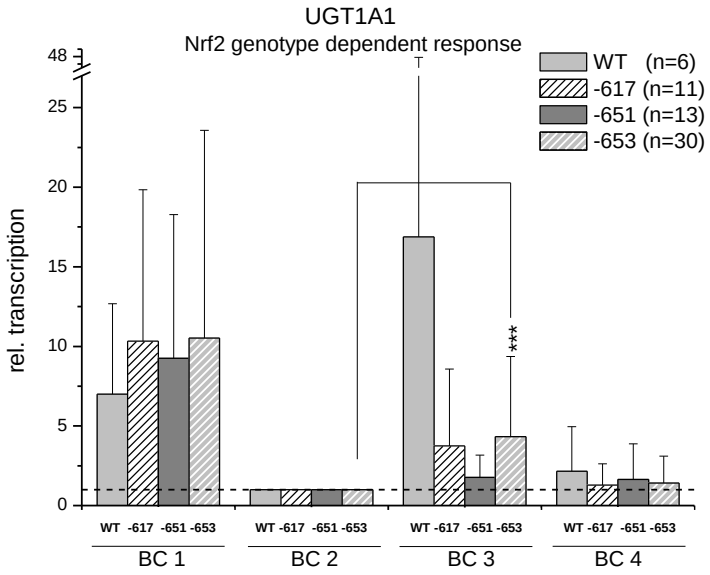

Figure Legends

Figure 1

(A) Modulation of Nrf2 gene transcription in PBLs of 49 participants of the intervention trial (samples of two participants failed to show sufficient fluorescence signals/ amplification). The data, analyzed in triplicates are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after four week wash out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750 mL coffee consumption (BC3), after a second wash-out period of four weeks (BC4). The data are represented as relative transcription of BC2=1. BC: blood collection. (B) Nr2 genotype dependent magnitude of changes in Nrf2 gene transcription during the course of the trial. The data are the mean ± SD of the individuals, carrying the respective genotype and presented as relative transcription of BC2 (wash out) =1. Significances indicated are calculated by Student's *t* test ($^{\#}p < 0.05$ and ***, $^{\#\#\#}p < 0.001$). BC: blood collection.

Figure 2

(A) Modulation of the GST1A1 gene transcription in PBLs of 48 participants of the intervention trial (samples of three participants failed to show sufficient fluorescence signals/ amplification). The data, performed in triplicates are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after four week wash-out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750 mL coffee consumption (BC3), after a second wash-out period of four weeks (BC4). The data are represented as relative transcription of BC2=1. BC: blood collection. (B) Nrf2 genotype dependent magnitude of changes in GST1A1 gene transcription during the course of the HSG trial. The data are the mean ± SD of the individuals, carrying the respective genotype and presented as relative transcription of BC2 (wash out) =1. Significances indicated are calculated by Student's *t* test (*, $^{\#}p < 0.05$ and ***, $^{\#\#\#}p < 0.001$). BC: blood collection.

Figure 3

(A) Modulation of the UGT1A1 gene transcription in PBLs of 46 participants of the HSG trial (samples of five participants failed to show sufficient fluorescence signals/ amplification). The data, performed in triplicates are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after four week wash-out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750 mL coffee consumption (BC3), after a second wash-out period of four weeks (BC4). The data are represented as relative transcription of BC2=1. BC: blood collection. (B) UGT1A1 genotype dependent magnitude of changes in UGT1A1 gene transcription during the course of the HSG trial. The data are the mean ± SD of the individuals, carrying the respective genotype and presented as relative transcription of BC2 (wash out) =1. (C) Nrf2 genotype dependent magnitude of changes in UGT1A1 gene transcription during the course of the HSG trial. The data are the mean ± SD of the individuals, carrying the respective genotype and presented as relative transcription of BC2 (wash out) =1. Significances indicated are calculated by Student's *t* test (*, $^{\#}p < 0.05$, $^{**}p < 0.01$ and ***, $^{\#\#\#}p < 0.001$). BC: blood collection.

Table 1

Gene	SNP	Frequency		Literature		
		n	[%]	n	[%]	
Nrf2	**-617C/C (WT)**	39/49	80	16/20	80	[17]
	-617C/A	10/49	20	4/20	20	[17]
	-651G/G (WT)	34/49	69	18/20	90	[17]
	-651G/A	15/49	31	2/20	10	[17]
	-653A/A (WT)	18/49	37	15/20	75	[17]
	-653A/G	31/49	63	5/20	25	[17]
GST1A1	**A/A (WT)**	24/48	50	106/27	38	[20]
	A/B	18/48	38	133/27	48	[20]
	B/B	6/48	12	39/278	14	[20]
UGT1A1	**$[TA]_6TAA$ (WT)**	21/46	46	183/399	46	[23]
	$[TA]_{6/7}TAA$	19/46	41	169/399	42	[23]
	$[TA]_7TAA$	6/46	13	47/399	12	[23]

Literature

[1] Prochaska H. J., Santanmaria A. B., Talalay P. (1992) Rapid detection of inducers of enzymes that protect against carcinogens. *Proc. Natl. Acad. Sci*. **89**, 2394-2398.

[2] Talalay, P., De Long, M. J., Prochaska, H. J. (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis.Proc. *Natl. Acad. Sci*. **85**, 8261-8265.

[3] Itoh K., Chiba T., Takahashi S., Ishii S., Igarashi K., et al. (1997) An Nrf2/small Maf heterodimer mediates the induktion of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and Biophysical Research Communications*, **236**, 313-322.

[4] Kwak M.-K., Itoh K., Yamamoto M., Kensler T. (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: Role of Antioxidant Response Element-like sequences in the *nrf2* promoter. *Molecular and Cellular Biology*, **22(9)**, 2883-2892.

[5] Li J., Johnson D., Calkins M., Wright L., Svendsen C., et al. (2005) Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicological Science*, **83**, 313-328.

[6] Chen C., Pung D., Leong V., Hebbar V., Shen G., et al. (2004) Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. Free *Radic Biol Med*. **37**(10), 1578-90.

[7] Kensler T. W., Wakabayashi N., Biswal S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47**, 89-116.

[8] Rushmore T. H., Morton M. R., Pickett C. B. (1991). The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *The Journal of Biological Chemistry*. **266**(18), 11632-11639.

[9] McMahon M., Itoh K., Yamamoto M., Chanas S., Henderson C., et al. (2001) The Cap ´n´ Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Research,* **61,** 3299-3307.

[10] Balogun E., Hoque M., Gong P., Killeen E., Green C., et al. (2003) Curcumin activates the Heme Oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. Journal*, **371**, 887-895.

[11] Yan H., Magilnick N., Lee C., Kalmaz D., Ou X., et al. (2005) Nrf1 and Nrf2 Regulate Rat Glutamat-Cystein Ligase Catalytic Subunit Transcription Indirectly via NF-kB and AP-1. *Molec. And Cellul. Biolo*. **25**(15), 5933-5946.

[12] Ishii T., Itoh K., Takahashi S., Sato H., Yanagawa T., et al. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem*. **275**(21), 16023-9 Hidgon, V., Frei, B. (2006) Coffee and health: a review of recent human research. *Crit. Rev. Food Sci. Nutr.* **46**(2), 101-123.

[13] Boettler U., Volz N., Pahlke G., Teller N., Kotyczka C., et al. (2011) Coffees rich in chlorogenic acid or N-methylpyridinium induce chemopreventive phase II-enzymes via the Nrf2/ARE pathway in vitro and in vivo. *Mol Nutr Food Res.* **55(5)**, 798-802.

[14] Volz N. (2010) Beeinflussung ARE-regulierter Phase-II-Enzyme durch Kaffee und ausgewählte Inhaltsstoffe (Dissertation). University of Vienna, Department of Food Chemistry and Toxicology. AC number: AC08464667

[15] Boettler U., Volz N., Teller N., Haupt L.M., Bakuradze T., et al. (2012) Induction of antioxidative Nrf2 gene transcription by coffee in humans: depending on genotype? *Mol Biol Rep.* [Epub ahead of print PMID: 22314914.

[16] Ferguson LR. (2009) Nutrigenomics approaches to functional food. *Journal of American Dietetic Association*. **109**, 452-458.

[17] Marzec J.M., Christie J.D., Reddy S.P., Jedlicka A.E., Vuong H., et al. (2007) Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *The FASEB Jounal*, **21**, 2237-2246.

[18] Yamamoto T., Yoh K., Kobayashi A., Ishii Y., Kure S., et al. (2004) Identification of polymorphisms in the promoter region of the human NRF2 gene. *Biochem Biophys Res Commun.* **321(1)**, 72-9.

[19] Siedlinski M., Postma D.S., Boer J.M., van der Steege G., Schouten J.P., et al. (2009) Level and course of FEV1 in relation to polymorphisms in NFE2L2 and KEAP1 in the general population. *Respir Res*. **10**, 73.

[20] Coles B.F., Morel F., Rauch C., Huber W.W., Yang M., et al. (2001) Effect of polymorphism in the human glutathione S-transferase A1 promoter on hepatic GSTA1 and GSTA2 expression. *Pharmacogenetics*. **11**(8), 663-9.

[21] Kalthoff S., Ehmer U., Freiberg N., Manns M.P., Strassburg C.P. (2010) Coffee induces expression of glucuronosyltransferases by the aryl hydrocarbon receptor and Nrf2 in liver and stomach. *Gastroenterology.* **139**(5), 1699-710.

[22] Monaghan G., Ryan M., Seddon R., Hume R., Burchell B. (1996) Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet*. **347**(9001), 578-81.

[23] Van der Logt E.M., Bergevoet S.M., Roelofs H.M., van Hooijdonk Z., Morsche R.H., et al. (2004) Genetic polymorphisms in UDP-glucuronosyltransferases and glutathione S-transferases and colorectal cancer risk. *Carcinogenesis.* **25**(12), 2407-15