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Published

2012

Journal Title

Molecular Biology Reports

DOI

10.1007/s11033-012-1547-6

Induction of antioxidative Nrf2 gene transcription by coffee in humans: depending on genotype?

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Abstract

The Nrf2/ARE pathway is a major cellular defense mechanism that prevents damage by reactive oxygen species through induction of antioxidative phase II enzymes. However, the activity of the Nrf2/ARE system is not uniform .Variability in response is presumed to be depending on to Nrf2 genotype.

We recently completed a human pilot coffee intervention trial with healthy humans, where large interindividual differences in the antioxidative response to the study coffee were detected In the present paper we address the question whether differences in the modulation of Nrf2 gene transcription, assessed as an induction of Nrf2 gene transcription by Q-PCR, might be correlated with specific Nrf2 genotypes. To date, 9 Single Nucleotide Polymorphisms (SNPs) have been identified in the Nrf2 (NFE2L2) gene. Two of these, the -617C/A and -651G/A SNPs are located within the promotor region and have previously been reported to influence the activity of the Nrf2/ARE pathway by reducing Nrf2 transcriptional activity. Sequencing of the critical Nrf2 gene promoter region not only confirmed the existence of these SNPs within the participants of the trial around the expected frequency (33 % carrying the -617C/A, 17 % the -651G/A and 56 % the -653A/G SNP) but also indicated reduced Nrf2 gene transcription associated with a normal diet if the SNPs at position -617, -651 or -653 were present. Of note, the data also indicated the study coffee to increase Nrf2 gene transcription even in SNP carriers. This further highlights the relevance of genotype-dependent induction of Nrf2 gene transcription that appears largely to be influenced by dietary factors.

Background

Reactive oxygen species (ROS) produced by xenobiotic compounds of exogenous and endogenous origin, are considered detrimental to mammalian cell integrity and are correlated with the development of life-threatening ailments ,including cardiovascular diseases and cancers. However, during evolution, mammalian cells have developed different defense systems to mitigate or repair ROS-induced cell damage. One pivotal mechanism is the induction of phase II detoxifying enzymes [1, 2]. Thus, a deficiency in phase II enzyme activity is associated with e.g. an enhanced risk of colon cancer [3, 4].

The presence of an antioxidant response element (ARE/EpRE), located in the 5'-flanking region of important phase II genes such as glutathione *S*-transferases (GST), γ-glutamate cysteine ligase (γGCL), NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1) [5-7] plays a critical role in the activation of the expression of these genes. ARE is activated via binding of the transcription factor nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2 (Nrf2 or NFE2L2) that is sequestered in the cytoplasm in quiescent cells. Activation by ROS or upstream protein kinases leads to the release of Nrf2, its translocation into the nucleus and the activation ARE-dependent gene expression [8-13]. However, the activity of the Nrf2/ARE (EpRE) system differs dramatically amongst humans. Biochemical studies have associated allelic variations in the Nrf2 promoter region with differences in the ability to increase antioxidative phase II gene transcription [14]. To date, 9 Single Nucleotide Polymorphisms (SNP) have been identified in the Nrf2 gene [14-16]. 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 attenuated binding of Nrf2 to the ARE (EpRE), resulting in decreased Nrf2-dependent gene transcription. Furthermore, a correlation between individuals carrying the -651G/A genotype and increased incidence of acute lung injury (ALI), a disease, related to reduced antioxidative cell defense has already been found [14].

Recently, we demonstrated that daily consumption of a special study coffee brew, enriched in various antioxidants for four weeks potently increased Nrf2 gene transcription in humans [17]. Yet, in both, this pilot trial, and an earlier coffee intervention trial [18, 19] large differences in the Nrf2 activation ability were detected amongst the individuals, which could not be explained by differential general health

status nor nutritional behaviour 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 [20]. Here, we investigated whether the interindividual differences in the response to consumption of a study coffee and elsewise normal diet could be explained by the presence of specific Nrf2 genotypes.

Methods

Materials and methods

Subjects

The test population comprised 18 male participants of the pilot coffee intervention trial performed earlier (figure 1) [19]. Originally, this intervention trial comprised thirty three probands. The here described investigations on the Nrf2 genotype of the individuals were performed more than one year after the trial and only 18 accepted to be genotyped. Exclusion criteria already applied in the original study were smoking, obesity (BMI>32), use of medication and chronic diseases. Participants had to keep their usual dietary habits for the duration of the study, except 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. Each participant was asked to apply the OG-500 Oragene®•DNA sample collection kit for saliva donation. Genomic DNA (gDNA) was purified following the manufacturer's instructions. Briefly, of the total 2 mL Oragene/saliva sample, 1 mL of sample was transferred into a 1.5 mL microcentrifuge tube, 40 µL of Oragene® Purifier was added and mixed gently by inversion. The remaining 1 ml was stored for later use. After 10 min incubation on ice the sample was centrifuged for 3 min at 15,000 × g (RT). The clear supernatant was transferred into a 15 mL centrifuge tube, 1 mL of 95% ethanol was added and gently mixed by inverting it 10 times. After a further incubation step (10 min, RT) the sample was centrifuged for 2 min at 15,000 × g (RT). The supernatant was discarded and the pellet mixed with 500 uL of 70% ethanol. The sample was incubated for 1 min at RT., and ethanol was then carefully removed without destroying the pellet. After completely drying the DNA pellet, the pellet was dissolved in 500 µL of distilled water. To fully re-suspend the DNA, a further incubation step for 20 min at 50 °C was added. After ethanol precipitation, the

final concentration of DNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware).

Sequencing

SNPs rs35652124, rs6706649 and rs6721961:

To amplify 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 AmpliTaq® to a total reaction volume of 20 µL. Amplification was performed on a Veriti^{TM} 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 Exo SAP-IT® PCR cleanup kit (Affymetrix/USB) according to the manufacturer's instructions. 5 uL of PCR product, 1 uL of Exo SAP-IT® and 4 uL dH_2O were carefully mixed prior to incubation at 37 °C for 15min, followed by 80 °C for 15 min and 4 °C for 3 min. DNA concentration was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware) to a concentration of 20 ng/uL DNA, 1uL of DNA was added to the sequencing reaction containing 5 uL of BigDye Terminator v3 (BDT v3.1, Applied Biosystems), 1.3 uL of each NFE2L2 primer (one forward and one reverse reaction), 3.0 uL of 5 x BDT v3.1 Sequencing buffer to a final volume of 20uL. Amplification cycles were as followes: 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 uL of iced cold 3M sodium acetate (pH 5.2) and 2 uL 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, 50 uL of 100% ethanol was then added and after repeatedly vortexing and re-spinning 10,000 g for 5 minutes, samples were incubated for 15 min at RT. The product was then precipitated by centrifugation at 10,000 x g followed by an incubation at 4 °C for 20 min. The supernatant was then 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 110 Speed Vac® (Savant) on high drying mode for 5 min. Samples were then resuspended in 15 uL dH_2O. The purified products were then either stored at -20 °C or directly added into a Micro Amp™ potical 96 well reaction plate (Applied Biosystems) to be analyzed on a 3130 genetic analyzer (Applied Biosystems).

Gene transcription by Q-PCR

Isolation of human peripheral blood lymphocytes (PBL), RNA extraction and Q-PCR were performed as previously reported [18]. Briefly, total RNA was extracted from isolated PBLs following the manufactures handbook of the RNeasy®Mini Kit (QIAGEN, Hilden, Germany). 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). A control without reverse transcriptase (-RT) was included for each dilution series. The primer assays used for the NF2L2 gene was: Hs_NFE2L2_1_SG, QT00027384. ß-Actin: Hs_ACTB_1_SG, QT00095431 (QIAGEN, Hilden, Germany). Primer concentrations and realtime 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.

Ethics

The study was approved by the ethics committee of the Landesaerztekammer Rheinland-Pfalz, Mainz, Germany (no 837.207.08(6204)) and an amendment, approved in May, 2010. Written consent forms were obtained from all subjects.

Results and Discussion

In this study, we examined diet-derived effects on the activity of the antioxidative Nrf2/ARE pathway and assessed these by Q-PCR. Nrf2 gene transcription was examined in 18 healthy male participants who followed the human pilot intervention

trial [19]. The study design [for details see 19] included an initial four-week “wash out” period where no coffee and a polyphenol poor diet were compulsory. This was followed by daily ingestion of 750 mL of the special study coffee, in three servings for four weeks. The coffee intervention period was followed by another four weeks of “wash out” (figure 1). Before and after each phase of the study Nrf2 gene transcript levels were quantified by Q-PCR. As summarized in figure 2, Nrf2 gene transcription was slightly increased after four weeks of coffee consumption (figure 2, BC3) in comparison to all other BCs, to the wash-out period (BC2), to the individual pre-study diet (BC1) and to the second wash out period after coffee consumption (BC4). However, a clear disparity in the response to coffee became apparent. While 36 % of participants (termed coffee responders, n = 6) displayed a ≥1.5 alteration of Nrf2 transcription after study coffee consumption in comparison to the wash-out period, with no change in gene expression compared to their normal pre-study statues, 64 % of the participants (n=12) demonstrated no change in Nrf2 gene transcription during the course of the study (defined as coffee non-responders).

To further analyze the modulation of Nrf2 gene transcription by dietary factors the participants were stratified according to their basal Nrf2 transcriptional status (BC1). As summarized in figure 2, B, large differences in the basal Nrf2 transcription level were apparent. From these stratified participants, only 23 % (n = 4) of the individuals displayed a ≥1.5 Nrf2 transcription at the BC1 time point. These participants were classified as normal diet responders. Of note, these participants showed a significantly decreased Nrf2 gene transcription after the study coffee consumption (BC3).In contrast ,participants with low basal Nrf2 transcription demonstrated an increase in Nrf2 transcription after study coffee consumption (figure 2, B). However, the demonstrated large differences in the Nrf2 transcriptional levels during the normal diet could not be sufficiently explained by the individual diet of these participants.

Taken together, this data demonstrates that participants in the study showed a large variability in their Nrf2 response to the study coffee. In addition, no major differences in age, sex, health status, ethnicity or diet were apparent amongst the study participants [19]. Therefore, the variability in response may be associated with the Nrf2 genotype, critical for the transcriptional activity of Nrf2. Furthermore,single nucleotide polymorphisms(SNPs) in the Nrf2 gene, affecting the Nrf2 activity seem to be widespread: the -617C/A genotype is found in 20% of the European (Caucasian) population, the -653A/G in 25% and the -651G/A genotype in 10% (table

1) [14]. Thus, such functionally relevant differences in the Nrf2 genotype amongst the study collective might critically influence the individual response to the study diet.

We therefore investigated, whether differences in the Nrf2/ARE-activation potential could be correlated to a certain Nrf2 genotype by determining the frequency of the -617C/A, the -653A/G and the -651G/A genotype in the participants of the trial (figure 3). As summarized in table 2, the -617C/A SNP was present in 6/18 or 33 % of the participants, whereas 56 % of the participants showed the SNP at position -653A/G. The -651G/A SNP was only observed in 17 % (3/18) of the individuals examined.. The frequency of the SNPs found within the study population is in agreement with findings of Marcez et al. (2007) who reported -653A/G as most common and -651G/A appearing only rarely in Caucasians. Overall, the frequency of the SNPs found in the present study appeared slightly higher than previously published data,but this may be due to the relatively low number of samples examined.

When considering a potential correlation of Nrf2 genotypes of the participants with their antioxidative response (Nrf2 gene transcription at BC3 ≥1.5), 50 % of the -617 WT carriers (participants carrying the wild-type sequence at position -617) showed increased Nrf2 gene transcription (≥1.5) after both their normal diet (BC1) and likewise, after four weeks of coffee consumption (BC3) when compared to the wash out period (BC2) (Fig 4 A). Only 33 % of the -617 SNP carriers demonstrated an increased Nrf2 gene transcription on normal diet. These findings are in line with Marzec et al. (2007) who reported an influence of the SNP-617 on Nrf2 gene transcription, resulting in a reduced transcriptional activity of Nrf2. Yet the frequency of -617 SNP carriers showing an elevated Nrf2 transcription increased by another 50 % after coffee consumption, indicating the potential of the study coffee to increase transcription, especially within carriers of this genotype.

We next examined the potential role of the -651C/A SNP on Nrf2 activation after coffee consumption. Of the coffee responders, 83 % carried the WT sequence, the same frequency as individuals carrying the WT and showing an increased Nrf2 transcription level upon their individual diet. Although none of the -651SNP carriers displayed elevated Nrf2 transcription at base, 17 % of these participants responded after coffee consumption with increased Nrf2 transcription. A reduced Nrf2 transcription activity of -651SNP has been previously reported [14] further supporting our results. The increase in Nrf2 transcript levels found in the present study even in

individuals carrying the -651C/A SNP after coffee ingestion suggests the study coffee to be an effective Nrf2 activator.

When we correlated the Nrf2 response with the presence of the -653A/G SNP, WT carriers demonstrated increased Nrf2 transcription after coffee consumption (50 %). In contrast, of the -653A/G SNP carriers only 12.5 % of the responders showed an increased Nrf2 transcription during their normal diet carried, which increased to 37.5 % after study coffee consumption. This is in contrast to Marzec et al. (2007) who failed to detect any effect of the -653A/G SNP on increased transcription of Nrf2.

We then examined the transcriptional changes of Nrf2 during the course of the trial for participants with Nrf2 gene transcription at base (BC1 ≥1.5) and correlated this with genotypes. As shown in figure 4, B the frequency of -617WT carriers who did respond to their individual diet was 75 %, whereas only 25 % of this group demonstrated any increase in Nrf2 transcription after coffee consumption. Of note, in these participants, the frequency of participants (25 %) carrying the -617SNP and acting as normal diet responders remained the same after coffee consumption. The same trend was apparent in the -651SNP.

In the group with enhanced Nrf2 transcription >1.5 at base (normal diet responders) the frequency of the -651WT was 100 %. Thus, all participants who showed a ≥1.5 level of Nrf2 transcription at BC1 carried the WT genotype. Interestingly, this was reduced to 50 % after the study coffee consumption. The -651SNP was not observed in normal diet responders at neither the BC1 nor BC3 time-points. Taken together, this seems to indicate, that under normal dietary conditions, -617WT and -651WT carriers have an elevated basal level of Nrf2 transcription, which does not appear to be affected by coffee consumption. Whether WT carriers for these two SNPs need, additional dietary or other factors to regulate their Nrf2 transcription levels is as yet unknown. When we examined the -653 SNP in these non-responders, (figure 4, B), the frequency of -653WT carriers as well as -653SNP carriers demonstrating a ≥1.5 increase in gene transcription remained the same with 50 % showing an increase after BC1 and 25 % following coffee consumption (BC3). As such, no -653 genotype dependent modulation seemed to be associated with this SNP.

The increased sensitivity of SNP carriers to coffee in comparison to WT is also apparent if comparing the magnitude of Nrf2 activation after coffee consumption. As given in figure 4, C WT carriers do not show any increase in Nrf2 gene transcription

after coffee consumption (BC3, 0.9 ± 0.2) whereas individuals, carrying at least one SNP display elevated Nrf2 transcript levels at (BC3), with -617SNP showing the strongest response in average (1.6 ± 0.5).

Supported by previous studies, our data indicate the presence of a specific Nrf2 genotype to be relevant for the regulation of antioxidative Nrf2 signalling [14, 16]. Interestingly, individuals carrying SNPs at positions -617, -651 or -653 displayed a reduced Nrf2 transcription at base under normal dietary conditions. However, the study coffee induced increased antioxidative Nrf2 transcription in these individuals. The finding that SNP carriers responded more strongly to the study coffee is further supported by the correlation of Nrf2 genotypes to previously examined changes of oxidation related biochemical parameters (Table 3).This relates to reduced spontaneous and oxidative DNA damage after coffee consumption in white blood cells as well as improvedf plasma glutathione status(GSH) and enhanced glutathione reductase activity [for detail see 19]. Carriers of the -651G/A seem to display enhanced sensitivity to the study coffee as demonstrated by increased Nrf2 transcription, marked reduction in DNA damage, enhanced increase in plasma GSH levels, altogether indicative for enhanced antioxidative cellular response [18, 19].

Conclusions

Our data indicate individuals carrying SNPs at position -617, -651 or -653 to be less likely to display an increased Nrf2 gene transcription at base, reflecting normal dietary conditions. In these individuals, consumption of a special study coffee resulted in an increased Nrf2 gene transcription. In contrast, WT carriers, demonstrated a higher level of transcription at base.in a situation where antioxidative food constituents are provided from various sources. To our knowledge, this study is the first to highlight the functional relevance of (a) specific Nrf2 genotypes to the activation of antioxidative Nrf2 gene transcription in healthy individuals and to (b) diet related modulation of Nrf2 gene transcription in individuals. Confirmatory studies with a larger population cohort are now under way to validate these exploratory findings.

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Figure Legend

Figure 1

Schedule of the human intervention trial. BC: blood collection.

Figure 2

Representative electropherograms of the amplified 424 bp region of the NFE2L2 promoter region (reverse strand shown and named in brackets). a) rs6721961: WT-617C(G); b) rs6721961: SNP-617A(T); c) rs6706649: WT-651G(C); (d) rs6706649: SNP-651A(T); (e) rs35652124: WT-653A(T); (f) rs35652124:SNP-653G(C). SNP: Single Nucleotide Polymorphism; WT: Wilde type.

Figure 3

Modulation of the Nrf2 gene transcription in PBLs of participants of the pilot coffee intervention trial, divided into (a) coffee responder and coffee non-responder and (b) normal diet responder and normal diet non-responder. The data, performed in duplicates 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.

Figure 4

Frequency of a Nrf2 genotypes in relation to the Nrf2 gene transcription level at base (BC1) or after study coffee consumption (BC3) in comparison to the wash out period (BC2), (A) coffee responders (n = 6) and (B) base level (n = 4). (C) Genotype dependent magnitude of 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.

Figures

Figure 1

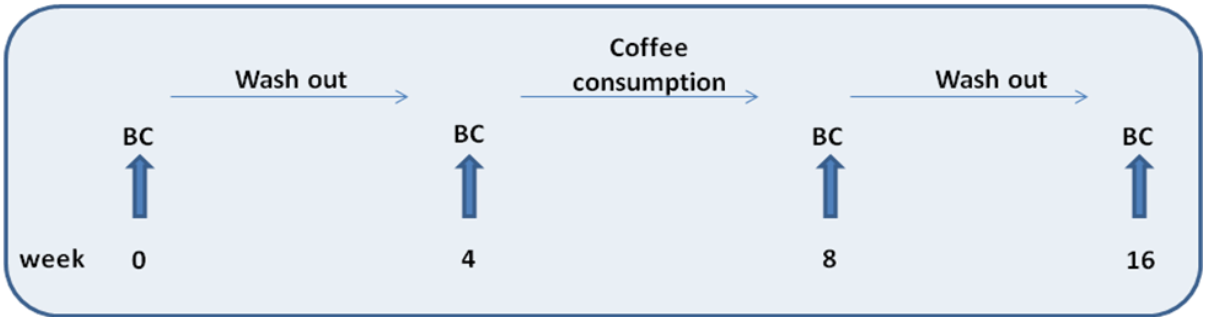


Figure 2, A

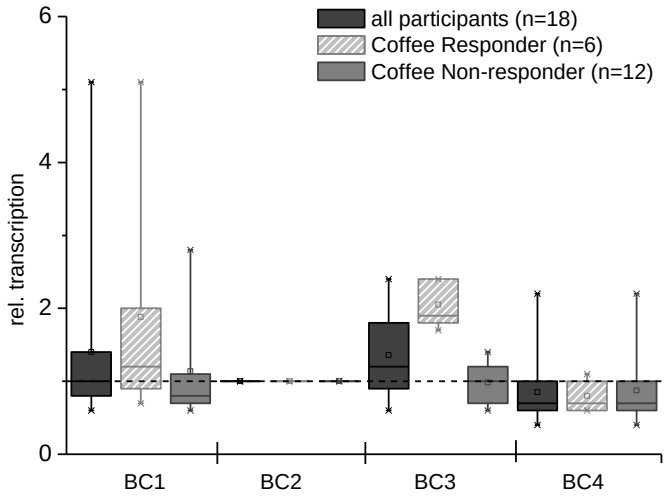


Figure 2, B

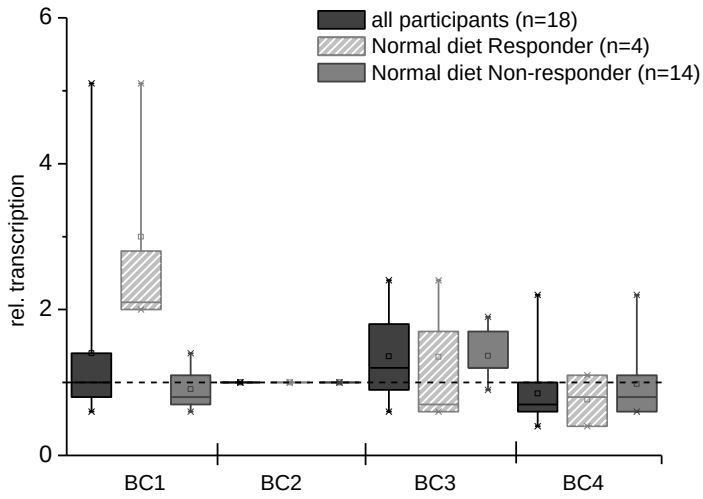


Figure 3

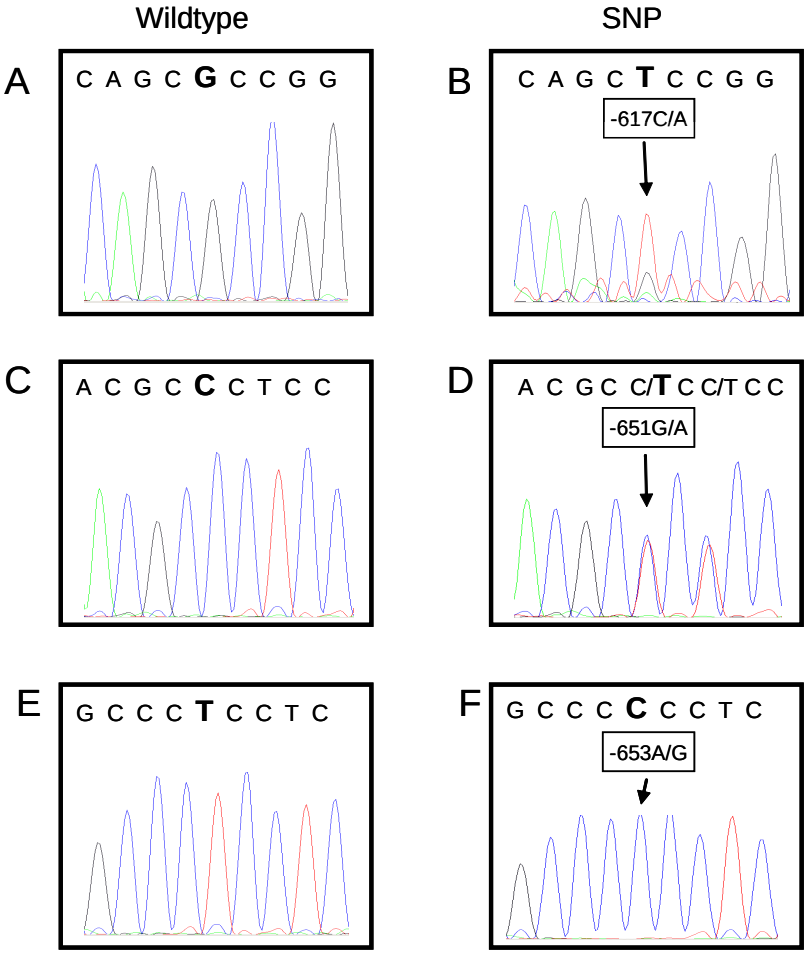

Wildtype
SNP
A
C A G C G C C G G
B
C A G C T C C G G
-617C/A
C
A C G C C C T C C
D
A C G C C/T C C/T C C
-651G/A
E
G C C C T C C T C
F
G C C C C C C T C
-653A/G

Figure 4, A

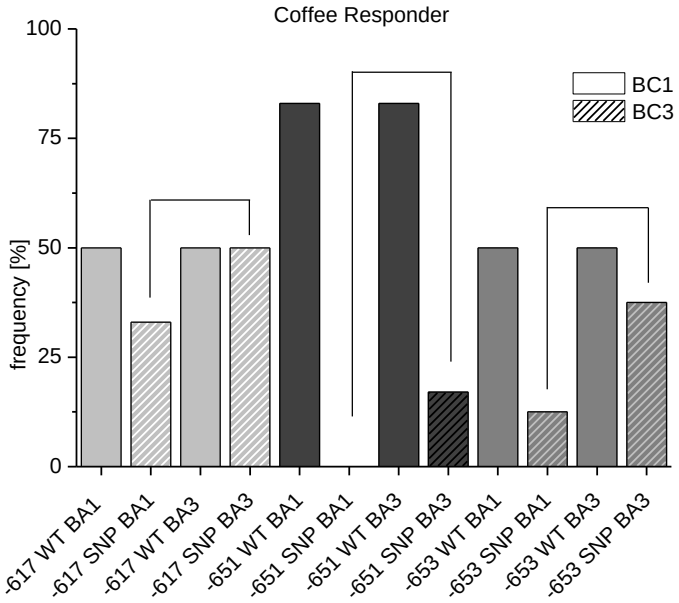


Figure 4, B

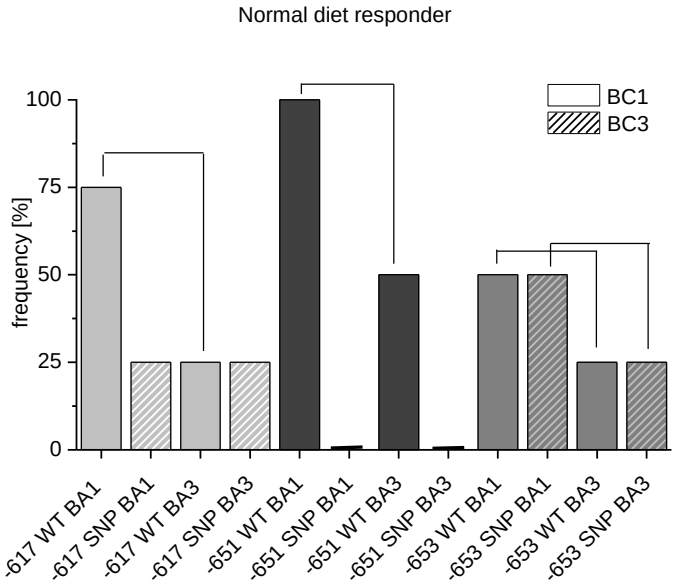
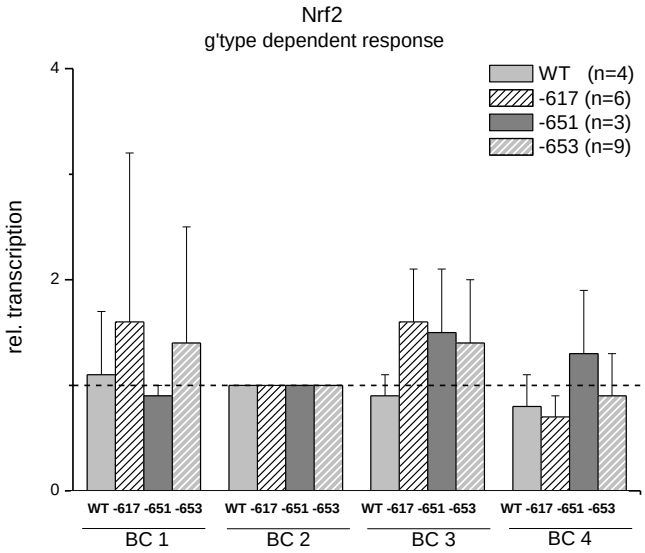


Figure 4, C


Nrf2
g'type dependent response
WT (n=4)
-617 (n=6)
-651 (n=3)
-653 (n=9)
rel. transcription
4
2
0
WT -617 -651 -653
WT -617 -651 -653
WT -617 -651 -653
WT -617 -651 -653
BC 1
BC 2
BC 3
BC 4

Tables

Table 1

SNP name	rs-ID	Position in Genome	Gene location	Alleles	Presence in Caucasians	Ref.
-617C/A	rs6721961	177838280	-617	C>A	20%	[14]
-651G/A	rs6706649	177838317	-651	G>A	10%	[14]
-653A/G	rs35652124	177838319	-653	A>G	25%	[14]

Table 2

Frequency of genotype in participants of the coffee intervention study

SNP name	Genotype	Frequency	
		n	[%]
-617C/A	CC	11/18	67
	CA	5/18	28
	AA	1/18	6
	Presence of A	6/18	33
-651G/A	GG	14/18	83
	GA	3/18	17
	AA	0/18	0
	Presence of A	3/18	17
-653A/G	AA	8/18	44
	AG	9/18	50
	GG	1/18	6
	Presence of G	10/18	56

Table 3

Correlation of the Nrf2 genotype to changes in Nrf2 gene transcription, oxidative DNA damage in white blood cells and the modulation of plasma glutathione (GSH) after coffee consumption (BC3) in comparison to the previous wash out period (BC2) [for detail see 19]

Genotype	Change of ox. DNA damage BC3 - BC2	Change of GSH level BC3 -BC2 [%]	rel. Nrf2 transcription BC3 - BC2
All (n=18)	2.2±1.3	111.9±10.3	1.4±0.6
no SNP (n=4)	1.5±1.0	114.0±7.6	0.9±0.2
617SNP (n=6)	2.5±0.6	108.2±12.9	1.7±0.5
651SNP (n=3)	3.3±2.1	115.6±1.9	1.5±0.6
653SNP (n=9)	2.4±1.5	110.2±11.8	1.4±0.6
617SNP+651SNP (n=8)	2.7±1.4	112.2±12.2	1.6±0.5
651SNP+653SNP (n=10)	2.4±1.4	111.0±11.4	1.4±0.5
617SNP+653SNP (n=12)	2.4±1.2	110.7±12.7	1.5±0.5
617-, 651-, 653-SNP (n=13)	2.7±1.4	111.3±12.1	1.5±0.6