Randomized controlled trial: effects of diet on DNA damage in heavy smokers

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We have conducted a randomized trial which investigated the ability of dietary changes (in particular diets rich in cruciferous vegetables and flavonoids), to increase urinary antimutagenicity and inhibit DNA damage in smokers. Ninety heavy smokers were recruited and randomly assigned to three groups, and were given three different diets. The first diet was based on flavonoid-rich foods, particularly cruciferous vegetables, but not based on supplementation; the second was a normal isocaloric diet (with an adequate administration of fruits and vegetables); and the third was based on supplementation of flavonoids in an adequate administration of fruits and vegetables); and

The investigations mentioned above were small-scale pilot studies. Here, we describe the results of a randomized trial we undertook to confirm the ability of different diets to increase urinary antimutagenicity and to inhibit the formation of DNA adducts in smokers.

Subjects and methods

Although the study design has been described in detail elsewhere (6), together with results concerning urinary antimutagenicity, we summarize it here conforming to the CONSORT guidelines (http://www.consort-statement.org).

Participants

This blind randomized controlled trial was conducted in Torino, Italy, among a population of healthy volunteers. An introductory phase (run-in), lasting 1 month, preceded the trial, consisting in a qualifying visit during which 120 volunteers were asked to complete an introductory questionnaire. Only healthy male heavy smokers of at least 15 cigarettes/day for the last 10 years, with average dietary habits, were admitted (vegetarians excluded). Inclusion criteria were verified and an informed consent form was submitted to the volunteers. The volunteers were asked to collect their urine and provide a blood sample. Only those subjects with a good adherence to the dietary guidelines (http://www.consort-statement.org).

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protocol at the end of the ‘run-in’ were included in the trial. Eventually, only 90 volunteers complied with the requirements, agreed to participate and were randomized into three groups.

Interventions
One diet was based on flavonoid-rich foods (particularly cruciferous vegetables), but not supplemented (‘flavonoid-rich’), one was a normal isocaloric diet (with an adequate administration of fruits and vegetables) (‘normal’), and the third was based on supplementation of the normal diet with additional flavonoids in the form of green tea and soy products (‘supplement’).

After the run-in phase, the 90 (unpaid) participants were taught how to prepare the food at a course with a professional cook. Participants were invited to entirely substitute their diet and in the morning (first urine after voiding) of the day and the DNA adduct levels in exfoliated cells at the end of the trial are expected to be representative of the events occurred during the trial. All participants filled a food frequency questionnaire (FFQ) at the baseline, and then started filling a dietary daily diary for the experimental month. An FFQ was filled again after 1 year since the end of the trial, and smoking habits were recorded again. Intake of flavonoids, several vitamins and folate were estimated through the self-administered daily diary, checked weekly and abstracted by a dietician. The dietician developed a food-nutrient-intake matrix specifically focused on flavonoids, to quantitatively assess the intake. Assessment was blind as to the group assignment.

Objectives and outcomes
The main objectives were an increase in urinary antimutagenicity (results described in ref. 8) and a reduction in DNA adducts in exfoliated bladder cells. Urine samples were collected on afternoons (between 4 and 10 pm) of the seventh day of diet and in the morning (first urine after voiding) of the day after, each week for 4 weeks. A further urine sample was collected from each participant a year after the end of the trial (both in the morning and in the afternoon) to measure DNA adducts in exfoliated bladder cells again. Urine samples were collected in polyethylene bottles which were kept at 4°C in the volunteer’s homes. The samples were collected weekly by the dietician who also verified protocol compliance. A part of each sample was filtered to collect exfoliated bladder cells for the measurement of DNA adduct levels. The remaining urine samples were kept at −30°C and analysed within 6 months. DNA adducts in the exfoliated bladder cells were measured with a 32P-postlabelling method in principle sensitive to aromatic adducts including polyaromatic hydrocarbons (PAH) and amines since no adduct enhancement method is employed, although each DNA sample is analysed with 200 mCi of 32P-ATP (2.9). Urine extracts for antimutagenesis studies were prepared using Bond Elut cartridges, according to a published procedure (10). The urinary antimutagenicity has been measured in S. typhimurium YG1024 in the presence of liver S9 from Sprague–Dawley male rats, treated with Aroclor 1254 (10). Blood samples were collected and the buffy coat was separated to measure bulky DNA adducts by P32-postlabelling in white blood cells (11).

Sample size
In the first pilot study (2), when the levels of DNA adducts in exfoliated bladder cells had been divided in tertiles, the urinary antimutagenic activity expressed as decrease in revertants/ml of urine equivalent was 21.3 in the tertile with the lowest level of DNA adducts, and 12.75 in the tertile with the highest level (a 40% reduction). In a further study (5), bladder cancer patients with a high consumption of fruits and vegetables showed in bladder biopsies a median concentration of 4-ABP adducts of 1.0/106, against the value of 4.0/106 found in subjects who did not consume fruits or vegetables. The standard deviation was 3. The sample size for the present study was estimated on the basis of the following assumptions: alpha error = 0.05, beta error = 0.20 (power = 80%); the alpha error expresses the probability of a chance finding (Type I error) owing to chance fluctuations related to sampling. The beta error (Type II error) is the complementary to 1 of the power. An average expected concentration of DNA adducts was assumed to be ~40% lower in subjects with higher consumption of flavonoids.

The applied formula is: \( n > 2(\frac{Z_{alpha}}{2} + Z_{beta})^2SD/D_0^2 \), in which the value of \( D_0 \) is the expected difference between the two arms. On this basis, we decided to recruit 120 subjects. With 90 subjects the power drops from 80 to 65%, other conditions being equal.

Randomization
A progressive number was assigned to each eligible volunteer. Randomization was performed by the project manager who randomly assigned the subjects to the three groups. Random numbers were generated by computer. The sequence was concealed until interventions were assigned. The selected volunteers were invited to participate in the study and to sign a consent form. To guarantee blindness in the subsequent course of the study, only the project manager was aware of the corresponding diet for each group.

Statistical methods
We computed means, medians and standard errors. In univariate analyses, we computed P-values from t-test for pairs, and P-values for trend from linear regression. Logistic regression analyses were performed to study the association between dietary changes and adduct levels. Estimates were adjusted by the number of cigarettes smoked at the time of urine collection (after 1 month and after 1 year). All analyses were conducted using the SAS package.

Results
Participant flow and baseline data
During the experiment 3 drop-outs occurred, corresponding to subjects who were not able to comply with the ‘supplement’ diet. Adducts could not be measured for technical problems (limited amount of DNA) in 17 of the remaining 87 subjects. Table I shows the baseline dietary habits for selected relevant food items (grams/day). Although some differences can be noted among the three groups, none is statistically significant.

Outcomes and estimation
Adducts were detectable at baseline in the exfoliated bladder cells of 68% of controls, 59% of the ‘flavonoid’ group and 65% of the ‘supplement’ group (\( P = 0.77 \)). We were not able to identify specific adducts since they often appeared as part of a diagonal zone of radioactivity on the thin layers.
The correlation coefficient between the number of cigarettes smoked and the sum of adduct levels in exfoliated bladder cells was 0.32 ($P = 0.14$). Table I shows estimated average (mean and median) daily intake of flavonoids by group and the corresponding DNA adduct levels (mean and median at baseline, after 3–4 weeks and after 1 year since the start of the trial). Weeks 0 and 1, and Weeks 3 and 4 were lumped together to increase power. The intake of flavonoids was considerably different between the first and the other two groups, with a dose–response relationship that was statistically significant (Table I). The number of cigarettes smoked was higher in the control group than in the treatment groups, probably by chance (i.e. in spite of randomization). This seemed to affect baseline DNA adduct levels, which were higher in the controls. Adduct levels increased in the control group after 1 month, and so did the number of cigarettes smoked.

After 1 month and after 1 year since the start of the experiment there was a decrease in total adduct levels in the ‘supplementation’ group. However, none of the associations was statistically significant, and the comparisons are hampered by the higher levels of adducts in controls at baseline. A slight decrease is observed also in bulky DNA adducts in white blood cells of the supplementation group after 1 month, but again it is not statistically significant. There was no association between urinary antimutagenicity and adducts levels.

Table II shows multivariate models that consider both the experimental groups and the intake of flavonoids as independent variables. Given the baseline differences in smoking habits, all comparisons are adjusted by number of cigarettes smoked (1 month) and median daily intake of flavonoids by group and the corresponding DNA adduct levels (mean and median at baseline, after 3–4 weeks and after 1 year since the start of the trial). Weeks 0 and 1, and Weeks 3 and 4 were lumped together to increase power. The intake of flavonoids was considerably different between the first and the other two groups, with a dose–response relationship that was statistically significant (Table I). The number of cigarettes smoked was higher in the control group than in the treatment groups, probably by chance (i.e. in spite of randomization). This seemed to affect baseline DNA adduct levels, which were higher in the controls. Adduct levels increased in the control group after 1 month, and so did the number of cigarettes smoked.

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Table II shows multivariate models that consider both the experimental groups and the intake of flavonoids as independent variables. Given the baseline differences in smoking habits, all comparisons are adjusted by number of cigarettes smoked at the time of urine collection. A statistically significant effect of flavonoid intake can be observed only after 1 year since the start of treatment ($P = 0.03$). Dietary changes were in fact still present at 1 year. In particular, after 1 year the intake of cruciferous vegetables was 6.7 g/day on average in groups ‘flavonoid-enriched’ and ‘supplementation’, versus 2.85 in the control group, and there were still statistically significant differences among the three groups for the intake of cauliflower and cabbages ($P = 0.02$), two of the main sources of flavonoids.

No adverse event was registered.

**Discussion**

**Interpretation**

We have found in our randomized controlled trial that dietary modifications using special recipes and instructions by a chef during an intensive course can affect the consumption of flavonoids and isoflavonoids (8). The intervention was focused on increasing the flavonoid/isorflavanoid intake, and it was successful in that respect. The intake of flavonoids was considerably different between the first and the other two groups,
with a dose–response relationship that was statistically significant. None of the other nutrients showed a similar pattern (6). Flavonoid intake was mainly related to the intake of cabbages (correlation coefficient 0.25; \( P = 0.02 \)) and fruit (0.27; \( P = 0.01 \)). The urinary concentration of phenolics was strongly associated with antimutagenicity (6). We tested multiple regression models with urinary antimutagenicity as the dependent variable, and dietary habits (fruit and vegetable intake) and level of urinary phenolics as independent variables (data shown in ref. 8). Only the urinary concentration of phenolics was associated with antimutagenicity. None of the dietary variables showed a correlation with antimutagenicity (8). Differences in flavonoid intake were appreciated between the first group (normal diet) and the other two (flavonoid-enriched and supplementation), suggesting that a dietary modification can be as effective as supplementation.

In the same study, we have now found that diets rich in flavonoids might modify the levels of DNA adducts in exfoliated bladder cells, but the evidence is equivocal. Total adducts were lower in the two treatment groups than in the control group already at baseline, possibly for the uneven distribution of smoking habits. In multivariate analysis, however, an effect of the increased intake of flavonoids on total adducts was suggested after 1 year since the start of the experiment, and was more evident than the difference among groups, after adjustment by cigarettes smoked at the time of urine collection. The fact that adducts after 1 year were lower in the experimental groups (particularly in the group with supplementation of flavonoids), and were influenced by flavonoid intake, is somewhat surprising. It could be owing to chance or could be attributed to the persistence of healthy habits in such groups.

The lack of a significant correlation between the adduct levels and the urinary mutagenicity is not completely surprising in light of the very different exposure intervals integrated by each of the markers. Urinary mutagenicity is an estimate of exposure during the very recent past, while the adduct levels in the exfoliated urothelial cells likely integrate exposure during an interval of \(~100\) days.

**Generalizability**

Although equivocal, our findings are in agreement with previous evidence suggesting complex biological effects of flavonoids. In particular, flavonoids can have a protective effect on adduct formation through several mechanisms, including inhibition of Phase I and induction of Phase II enzymes (12,13). The interpretation of our study is limited by the lack of correlation between changes in flavonoid intake and levels of urinary phenolics (8). Bioavailability of flavonoids could be questioned, according to previous findings in which the majority of the flavonoids are degraded within the gut by intestinal bacteria and do not reach the systemic circulation (14). However, our study suggests that an increased intake of plant foods can modify the adduct levels, whether or not this is due specifically to flavonoids.

Many studies have suggested that the Mediterranean diet, and generally a high consumption of cereals, fruits and vegetables, reduces the risk of cancers at different organ sites, including the colon, breast, bladder and prostate (1,11). The Mediterranean diet plays a protective role in cardiovascular disease. Different components of this diet have attracted attention, especially tomatoes and olive oil. Flavonoids are a particularly relevant group of polyphenols which have been suggested to interfere with biochemical events involved in atherosclerotic pathology and cancer. A high concentration of flavonoids and other polyphenols has been measured in onions, lettuce, red wine and other elements of the Mediterranean diet. The ability of a diet rich in flavonoids to reduce the levels of smoking-related DNA adducts in exfoliated bladder cells could explain the lower incidence of cancer associated with Mediterranean diet. However, given the complexity of dietary patterns, and the equivocal nature of some of our findings, this is still a hypothesis that needs confirmation. Smoking is the most important single preventable cause of cancer; at the present stage of knowledge it is totally unlikely that certain dietary habits can seriously counteract the effects of tobacco smoking.

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**References**


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