FUNCFOOD publishable final report accumulated over 54 month

IMPACT OF AGENTS WITH POTENTIAL USE IN FUNCTIONAL FOODS ON BIOMARKERS FOR INDUCTION OF AGE RELATED DISEASES

This project represents a collaborative research project involving five European institutions that have been given support by the EU Directorate for Research, Brussels, and it also includes three different Indian research centres financed by the Department of Biotechnology, Government of India.

The challenge in this research is to investigate dietary factors, e.g., so called functional foods, that can prevent age related disease such as cancers, cardiovascular diseases and diabetes, which are the major causes of morbidity and mortality. Even a modest decrease in the prevalence of these maladies translates into millions of lives saved. “Functional foods” are commonly defined as foods that enhance the health benefit from diets beyond the traditional nutrients they contain by means of modification or addition of active ingredients.

The project´s overall strategy involves primary screening of putative protective compounds in cultured mammalian cells, where the most promising candidates are subsequently tested in animal models. In the final stage, compounds that on basis of the experimental data as well as evidence from the literature, seem most likely to prove beneficial will be investigated in humans. The age related diseases mentioned above take decades to develop, far longer than the four years allocated to this project. For this reason the studied effects will be monitored by means of “biomarkers”, analogous e.g. to the use of specific biological factors in blood and urine to detect development of disease.

The project concerns 15 separate working packages of which 5 are allocated to 3 different Indian Research Centres.

Work package 1 (WP1) was to provide the partners with a minimum of 11 presumptive protective agents from a candidate list for testing of protective effect. The list was including the following agents which were provided to the participating partners:

1. Astaxanthin (>97,1%)
2. Blueberries, freeze dried powder (anthocyanidines)
3. Butylated hydroxyanisole (BHA) (minimum 90% 3‑isomer 9% 2‑isomer)
4. Carnosine (>99%)
5. Chlorophyllin, commercial grade
6. Co‑enzyme Q10 (>98%)
7. Ellagic acid (>95%)
8. Gentiana lutea methanol extracts, from the Vinča Institute of Nuclear Sciences, Belgrade
9. Lycopene extract (75% lycopene and 10% beta-carotene)
10. Silibinin (>98%)
11. Teaphenon E (green tea polyphenols, 81,7% EGCG, 12,5% ECG, 2,5% EC, 3% being other tea components)
12. 6‑Gingerol (>98%)
13. Resveratrol (>99%)
14. Turmeric (70% curcumin)

Work package 2 (WP2) concerned the impact of the 11 protective agents on genotoxic effects in metabolically active human cells using human carcinogens to be the test agents to be a protection against. Two methods were used for screening, the DRAG assay and the *hprt* mutation assay.

Astaxanthin, blueberry extract, ellagic acid, Co-enzyme Q10, teaphenon E, chlorophyllin, Gentiana lutea extract, and 6-gingerol were protective in either of the methods used for genotoxicity. No protective effect was found with BHA, lycopene, resveratrol, silibinin and curcumin.

The Work package 2 (WP2) also included cytogenetic studies in vitro. Chromosome fragments which are lagging during cell division will be converted into micronuclei, the frequencies of which reflect the frequencies of induced chromosomal breaks. The results on micronuclei induction in vitro in HepG2 cells were considered protective when the reduction in the frequency of micronuclei was not associated with a significant increase of cytostasis. The protective compounds selected on the basis of screening were chlorophyllin, ellagic acid, blueberry, Gentiana extract and 6-gingerol.

Finally, the protective agents to be selected for the potential to be further tested *in vivo* were; astaxanthine, freezed dried blueberries, chlorophyllin, CoQ10, ellagic acid, Gentiana extract, 6-gingerol and theaphenone.

Work package 3 (WP3) concerned the impact of protective agents on cytogenetic parameters in Swiss albino mice. Chlorophyllin (CHL), Ellagic Acid (EA) and Blueberries (BB) were selected for testing based on the results in WP2. The results were considered remarkable when the reduction in the frequency of micronuclei was not associated with a significant effect on proliferation measured as % polychromatic erythrocytes (PCE).

Effect of protective agents against MNNG-induced DNA damage: CHL, EA, BB were effective in reducing the frequency of micronuclei. The effect of protective agents against DMBA-induced DNA damage: CHL and EA had no protective effects, on the contrary resulted in higher toxic effect. BB had a weak protective effect.

Work package 4 (WP4) concerned the impact of protective agents on multiple cellular/molecular targets during rodent carcinogenesis. This WP was reported only to the research unit DBB, India.

Work package 5 (WP5) concerned the impact of protection of carcinogenesis and other toxic effects in the rodent. This WP was reported only to the research unit DBB, India.

Work package 6 (WP6) concerned establishing procedures for sampling of blood, buccal cells and urine from humans to be used for WP 7 and 8.

Permits - From the Serbian Ministry of Health, the Vinča Institute has obtained permits to export human samples to the EU partner laboratories. Each package must be subject to a safety inspection to ensure that it does not contain infective or toxic materials.

Shipping **-** Frozen samples with appropriate documentation (permit obtained from Ministry of Health and Customs declaration specifying that the samples are not commercial) would be shipped on dry ice using express air service to the partner laboratories (BIU - Germany; Karolinska Institute, Sweden).

Work package 7 (WP7) concerned the dosing schedules for PAH intakes in humans. To set up adequate conditions for preparing meat with high levels of PAH; dosing regimens.

Extensive pilot experiments with grilling over an open fire of birch or fire wood using two types of grills have been conducted in order to obtain sufficiently high levels of PAH while retaining palatability. Pieces of pork as well as Serbian sausages (ćevapčići) were put on the rack of the grill and placed at various distances from the burning wood. The meat was grilled

to medium or well done for various time periods (3-20 min) and distances from the fire. The PAH profiles from the grilled meat samples were almost identical with that obtained by partner 3 under the 6th Fp project "Dietary exposure to PAH and DNA damages" (DIEPHY; FOOD-CT-2003-505609). The total concentration of 28 individually determined PAH after eating the sausages were in the range 1100 to 3200 µg/kg, with benzo[*a*]pyrene levels of 53 to 158 µg/kg in the sausage?.

Work package 8 (WP8) concerned voluntary humans having a meal of grilled sausages with high levels of PAHs, with and without combination of an protective agent, followed by analysis of the incidence of Lymphocyte Micronuclei, Comet Tail Intensity (% DNA in the tail), concentration of the oxidative stress marker 8-oxo-dG (nM), analysis of CoQ10 and α-tocopherol in blood and metabolites in urine.

Urine sampling: Spot urine samples of non-smoking study participants were taken on days 1, 2, 3, 5 and 11 (see WP7) and frozen at -20°C. After shipment to BIU on dry ice the samples were stored at -20°C until used for analysis. To allow the determination of urinary biomarkers of aromatic amines in the 3rd and 4th Serbian grilled meet study spot urine samples of non-smoking study participants were taken in the presence of *tert.*-butylamine which was used as required intercept-reactant.

Verifying smoking status: Cotinine is a longer-lived metabolite of nicotine and considered as biomarker of tobacco smoke exposure. Its urinary concentration was used to verify self-reported smoking status indicated by the study participants at Vinča Institute in their questionnaires. Cotinine was determined in each urine sample. Subjects with a cotinine level below 5 µg/L were classified as non-smokers, those with concentrations in the range of 5–150 µg/L indicated exposure to ETS, and those with levels above 150 µg/L were considered to be active smokers.

The entire grilled meat study to investigate the influence of antioxidants on the urinary biomarker levels (1st to 4th part) was performed at Vinča Institute in Belgrade. The PAH exposure levels associated with the grilled meat consumed by the study participants were also determined at Vinča Institute.

After a two weeks of wash-out period with no grilled meat consumption the treatment protocol with grilled meat was repeated for the same study group of 11 volunteers, but in addition all participants received a total daily dose of 3 x 200 mg chlorophyllin (CHL with Zn) for 8 consecutive days beginning 4 days before the PAH exposure period. The time course of the median values of the determined 5 isomeric OH-PHE, 1-OH-PYR, and Di-OH-PYR was investigated.

Results of the 1st Cohort: Chlorophyllin: Statistical evaluation of data has shown a statistical significant difference between a baseline level of lymphocyte micronuclei and SSBs (measured by comet assay) and the level after consuming the grilled meat with high content of PAHs. Pre-treatment with CHL restores both, the MN and SSBs level on the baseline value, and no further increase was observed while grilled meat was consumed. No correlation was found between lymphocyte micronuclei, Comet tail intensity and serum concentration of 8-oxo dG. Concentration of cholesterol increases with respect to PAH intake, concentration of coenzyme Q10 has not been changed, whereas a-tocopherol was lowered with respect to PAH intake. Pre-treatment with CHL significantly lowered not only cholesterol, but Q10 has and a-tocopherol, and in general is seen as a toxic effects of CHL supplementation (Life extension product). At the end of treatment (PAH intake + CHL) values of CoQ10 and α-tocopherol were recovered and reached higher values compared to base-line state, whereas concentration of cholesterol still was increased.

Without CHL a typical increase of all phenolic PAH exposure biomarkers are observed during the 4 days of PAH exposure period with an almost constant urinary elimination of the OH-PHE at day 3 and 5 and a maximum elimination for 1-OH-PYR on day 5, which was expected when an almost similar dose of total PAH to each participant is applied per day.

In the study with CHL as micronutrient the effects probably strongly depend on the tissue-localized CHL concentration. It appears to be likely that the protection of CHL against

micronuclei formation in lymphocytes after consumption of grilled meat is a specific effect against genotoxic PAH metabolites in these target cells but does not necessarily provide a general protection of all target organs.

Results of the 2nd cohort: Blueberry: Evaluation of data has shown slight increase of lymphocyte micronuclei after consuming the grilled meat with high content of PAHs. Pre-treatment with blueberry reduced the MN incidence above the baseline level. Blueberry supplementation slightly reduce the yield of PAH induced micronuclei as well as SSBs, but their level was significantly higher when compared to baseline state. In contrast to lymphocyte findings, micronuclei in buccal cells were independent of any treatment. Comet data have shown no significant difference between baseline level and level after PAHs ingestion. No correlation was found between level of lymphocyte micronuclei, as well as Comet tail intensity and serum concentration of 8-oxo dG.

Concentration of cholesterol increases with respect to PAH ingestion, whereas concentration of CoQ10 and a-tocopherol decreased with respect to PAH ingestion. Pre-treatment with blueberries almost restore concentration of CoQ10 and a-tocopherol on base-line level whereas cholesterol still was above base-line values. CoQ10 as well as a-tocopherol continue to decrease during second phase of eating regime where PAH enriched food was ingested together with blueberries. In conclusion blueberries display good antioxidant properties (seen as restoring of CoQ01, α-tocopherol, and micronuclei) but not enough to protect from the effect of PAH in grilled meat.

In the study with BB supplementation two interesting observations are made. First, after intake of BB during the pre-treatment phase of 4 days the control values of all urinary PAH exposure markers are substantially lowered on the first day before consumption of grilled meat starts if compared to those in the control study given no BB. This effect correlates nicely with the reduced rate of micronuclei formation detected in lymphocytes. Second, in general the excretion kinetic of the phenolic PAH biomarkers are modulated substantially. The maximum urinary levels are observed for most PAH exposure biomarkers after 2 days of ingestion of grilled meat and at day 5 the levels are reduced by 20-50% compared to the control group without BB supplementation. BB contains a complex mixture of various groups of polyphenolic antioxidants including anthocyanides, flavonoids such as quercetin, ellagic acid, resveratrol, and several pterostilbenes. Overall the bioavailability of most of these compounds which are resorbed predominantly as their glycosides is very low, for some of which less than 1% of the administered dose. However, the present data on the excretion of phenolic PAH exposure markers indicate that overall a substantial direct or indirect inhibition of cytochrome P450 including CYP1A1, 1A2, and 1B1 competent for PAH metabolism and/or phase 2 enzymes such as UDP-glucuronosyltransferase and sulfotransferases occurs in vivo by BB supplementation.

Results of the 3rd Cohort: Gentiana lutea: Evaluation of data has shown that PAH enriched grilled meat significantly increase the yield of lymphocyte micronuclei as well as yield of SSBs. Pretreatment with *Gentiana lutea* (capsules, 4x400mg per day) reduced the MN incidence for 30%. The incidence of lymphocyte MN continue to decrease when grilled meat was consumed together with *Gentiana* for further 30.27% and in 9 out of 12 persons reached zero value. Significant portion of apoptotic binucleated cells were observed, indicating that treatment with *Gentiana* induce apoptosis of damaged cells. The Comet tail intensity, i.e, percentage of the DNA in the tail follow the same trend as micronuclei and decrease during treatment, as well as the incidence of MN in buccal cells. Concentration of serum 8-oxo dG Observed concentrations positively correlates with cytological findings of apoptotic and necrotic lymphocytes.

Without GL root powder an increase of all isomeric aromatic amines of naphthalene and biphenyl was observed during the 4 days of ingestion of grilled meat. This clearly indicates that grilled meat is indeed a source of human exposure to these arylamines. However it should be pointed out that the excretion level of these amines is in the ng/L range indicating that they are occurring in much smaller levels in grilled meat compared to PAH. A maximum urinary elimination was noted for most arylamines at day 2 after 24 hours of ingestion of grilled meat

and all arylamine levels were higher after the wash-out phase at day 11 than the control value at the 1st day except for 2-ABi.

In the study with GL root powder no clear effects are observed on the excretion kinetic of the aromatic amines. Similarly also no clear effects are observed for the rates of urinary excretion of *o*-toluidine after consumption of grilled meat and GL root powder.

Results of the 4th cohort: Lycopene - Tomato juice: Evaluation of data has shown that PAH enriched grilled meat significantly increase the yield of lymphocyte micronuclei as well as yield of SSBs. Pre-treatment with Tomato juice (2x250ml per day) reduced the MN incidence for 26.6 %. When tomato juice was consumed together with PAHs enriched grilled meat the incidence of MN further increase for 8.1%.The similar results were obtained when the Comet tail intensity data were evaluated. The incidence of micronuclei in buccal cells didn't show any difference in respect to applied dietary regimes. Serum concentration of 8-oxo dG was not significantly enhanced with respect to ingested PAHs. Interesting finding was that pre-treatment with tomato juice reduce serum concentration of 8-oxo dG in statistically significant manner ($p<0.03$).

Without Tomato juice (lycopene) a typical increase of all phenolic PAH exposure biomarkers are observed during the 4 days of PAH exposure period with a maximum urinary elimination of the OH-PHE and OH-PYR at day 2 after 24 hours of ingestion of grilled meat except for 1-OH-PHE for which a maximum is observed at 3rd day. Overall the excretion kinetic of the 4th study group (reference grilled meat) looks very similar to that also observed for the 3rd study group with slightly lower total levels of the phenolic PAH metabolites

Taken together the obtained data after consumption of grilled meat as a source of PAH exposure and Tomato juice support the concept that phase 1 enzyme inhibition and phase 2 enzyme induction are the underlying chemo-protective mechanisms of lycopene against PAH-induced toxicity.

In the study with Tomato juice a substantial reduction in the maximum excretion levels of the aromatic amines could be detected. However, the effects of lycopene on the metabolism of carcinogenic aromatic amines such as *o*-toluidine, 2-ANa and 4-ABi disserve more detailed studies.

Evaluation of all results in WP8: According to findings in all cohorts it can be concluded that chlorophyllin, blueberries and tomato juice acts as a good antioxidants reducing PAH induced lymphocyte findings up to 30% on the average. The results of urinary metabolites supported those findings. *Gentiana lutea* did not behave as an antioxidant, it affected mitotic spindle, induced mal segregation of chromosomes, apoptosis and necrosis. It should not be recommended for intake by healthy humans.

Work package 9 (WP9) was to investigate the protective effect of 5 selected agents in a cohort of totally 25 smokers. The main objective in this work package was to recruit and perform intervention treatments of protective agents on voluntary smokers with a minimum daily consumption of 15 cigarettes. Since Sweden has the lowest prevalence of smokers in the western world, it has been difficult to identify and recruit a sufficient number (40) of smokers with a minimum daily consumption of 15 cigarettes. Finally 28 smoking persons was recruited and intervention studies with 5 protective agents have been completed. The studies were conducted in three different treatment periods; protective agent, placebo and wash out (see figure below). Sampling of blood and urine were taken before and after each period and thereafter distributed to the different laboratories for analysis. Each volunteer were investigated in terms of different life style factors such as general health status using standard clinical markers, questionnaire for eating habits, health profile, micronuclei in young erythrocytes (MN), oxidative stress marker (8-OxodG), haemoglobin adducts, urinary metabolites, endogenous levels of vitamin E and Q10.

The effect of antioxidants on frequency of micronuclei. The first supplement involved 5 volunteers having freeze-dried blueberries, organic certified and harvested in Scandinavia. The intake was 4 g/day which correspond to 28 g fresh blueberries containing about 200 mg anthocyanines.

The second supplement was chlorophyllin, an approved food colouring agent (E141). The intake was 200 mg/day. The dye is water soluble and with a similar structure as chlorophyll. It can inhibit binding of reactive molecules to cellular macromolecules by sequestering mechanism. Five volunteers was used to investigate this supplement.

The third antioxidant was Astaxanthine, a carotenoid produced by algae. It is produced as an oil emulsion when used as dietary supplement. The dose to 6 volunteers was 8 mg/day. Capsules containing lycopene extract from tomatoes was given as the fourth antioxidant to 5 volunteers. It provides the synergy of the capsules of extracts of tomato, phytoene, phytofluene, beta-carotene, phytosterols and vitamin E. One capsule corresponds to approximately 15 mg lycopene.

The fifth and last antioxidant was an endemic herb from central and southern Europe, Gentianella lutea. It is traditionally used as a flavour used in beverages in Serbia. The preparation was ingested by 6 volunteers taking one capsule/day containing 400 mg/capsule.

The protective effect in the intervention studies was compared to the initial effect of smoking habits of each individual. As compared to non-smoking references, the effect of smoking on MN levels was significant on average in the group. However, at an individual basis, there was no correlation between the number of cigarettes per day, which the volunteers had indicated in the questionnaire, and the individual level of MN. As will be discussed under evaluation of all data, it can be concluded that the MN level is depending on several life style factors. The sum of these are expected to be seen in the initial level of MN and, therefore, only the results of the antioxidants (AO) versus the initial MN-level.

The statistical analysis of 5 compounds suggest that chlorophyllin, freeze dried blueberries and astaxanthine have a protective effect against the genotoxicity in red blood stem cells of smokers. The overall results of the 5 antioxidants clearly showed that the effect of the antioxidants can only be detected when the individuals have elevated background levels of MN. The level does not necessary be the consequence of smoking, which is supported by the fact that there were no correlation between number of cigarettes per day and MN level. The results indicated that the effect of the antioxidant stayed for approximately 3 weeks, where after it started to fade off during the three placebo weeks and further during the washout period.

Analysis of protein adducts and the effect of antioxidants. Hb adducts is a marker of internal dose of electrophilic compounds/metabolites. The measurement of Hb adducts is based on detachment of adducts to N-terminal amino acids with Edman reagents and analysis by mass spectrometric techniques. Acrylonitrile (AN) is marker of tobacco smoking. Ethylene oxide (EO) is related to tobacco smoking, but there is also a low background in non-smokers due to endogenous formation. Acrylamide (AA) is a marker of tobacco smoking and also of intake of certain types of food. Glycidamide (GA) reflects metabolic activation of acrylamide.

The work started with different development work which concerned establishment of repeatability in quantitative analysis with the new GC-MS/MS instrument etc. in the analysis of adducts including test analysis of samples from earlier project; establishment of analysis of AN adducts and discovery and characterization of a side-reaction leading to the transformation to AA adducts; development of faster work-up of samples for the N-alkyl Edman method; switch to the use of the Fire procedure and evaluation of the performance of precise quantitative analysis by LC-MS/MS; testing of developed adductomics approach for analysis of unknown adducts.

The main result concerning the intervention with protective agents was that no obvious effects were detected. No change in adduct levels also means that the internal dose is about constant over time. In this case one could assume that this also means that the smoking habits have not varied over the time for the intervention. This would imply that if there is any difference in genotoxic effect during the intervention, it is not dependent on difference in exposure from tobacco smoke during the experimental period.

The adduct levels as expected are clearly increased in the smokers compared to non-smokers. Though, surprisingly there was no clear correlation of adduct levels with number of smoked cigarettes per day. In several published early studies with measurements of these

adducts from EO, AA and AN there have been clear correlations. This most probably tells that the inhalation exposure from the cigarettes varies a lot, maybe due to different types of cigarettes (development of filters etc.) and variation in inhalation habits. However, the reliability in the adduct analysis could be seen also from the linear regression between different adduct levels. A comparison with cotinine (analysis by Partner 3) gives a good correlation only when compared with the total adduct level (AN+EO+AA+GA adduct levels), but not with the specific adduct levels.

All samples analysed for the known adducts were also analysed for the 19 unknown adducts detected in the parallel adductomics project. The unknown adducts were detected in all blood samples analysed from WP9. The levels were similar to those in samples analysed in an earlier study. Evaluation is not complete, however, but no obvious trends related to the intervention were observed.

Analysis of 8-hydroxy-2'-deoxyguanosine (8-OxodG) and the effect of antioxidants. For studying antioxidant effect of components at the level of 8-oxo-dG (a stress biomarker), the blood samples were taken before and after 20 min extensive cycling using a trainee cycle. The cycling and blood collections were repeated at the three different treatment periods mentioned above (protective agent, placebo and wash out).

It has been suggested that dietary antioxidants reduce the level of oxidative DNA damage induced by reactive oxygen species. The SU-group has previously set up and published an in vivo method for analysing the antioxidant effect of a dietary compound. In the previous study we aimed to investigate the protective effect of tomato juice intake towards ROS induced by 20 min of extensive physical exercise. SU group has developed a sensitive ELISA method for detection of 8-oxo-dG in human blood serum as a marker of oxidative stress. In the previous intervention study we found that the level of 8-oxodG in human blood serum was increased significantly after 20 min severe physical activity possibly caused by an increase of the intracellular ROS level in the non-vegetarian and non-frequently trainers healthy donors. No increase was observed when individuals were drinking 150 ml tomato juice per day during a period of 5 weeks suggesting that the intracellular nucleic acids (particular in the nucleotide pool) were unaffected and well protected from ROS. The intervention study support the hypothesis that antioxidants (e.g. lycopene) supplied from tomato juice may protect against oxidative stress induced by extensive physical exercise.

Same experimental set up was used in the FuncFood project to test antioxidative effects of lycopene in capsule (20 mg/day), blueberry, astaxantin, gentiana and chlorophyllin. In this study 5 healthy smokers were asked to take antioxidants (AO) for periods of 5 weeks as follow: 5 weeks with AO, 5 weeks Placebo and 5 weeks without AO/placebo. Blood samples were collected before and after start of each period during the intervention study. At the day for blood collection, the individuals were asked to do 20 min physical exercise with 80% max pulse using a stationary motion cycle. To calculate the individual maximum pulse, the following generally accepted formula was used: 220 - age = maximum pulse. Blood samples were collected before and after exercise. The serum level of 8-oxodG was analyzed as a marker of oxidative stress. The results suggest that there were no protection of oxidative stress in the intervention of the tested antioxidants with the exception of blueberry. However, it is important to mention that even we do not see any significant effects of several of studied antioxidants in the present study we cannot conclude that they do not have any antioxidative effect. There are several reasons for this statements, firstly the study cohorts (5 donors per compounds) seems not to be an adequate number of individuals, secondly based on the results from other studies, only persons with high levels of oxidative stress have benefit from antioxidant supplementation and thirdly, some difficulties were found to handle some of the samples. It cannot be concluded which reasons is causing the present negative results.

Analysis of urine metabolites and the effect of antioxidants. Urinary PAH biomarker analysis in Swedish smoker and the influence of antioxidants have started during the period. For the polycyclic aromatic hydrocarbon (PAH) biomarker analysis in urine the well-established GC-MS method at BIU has been used. The following phenolic PAH metabolites have been determined in urine of the Stockholm smoker study group as PAH exposure markers: 1-, 2-, 3-

, 4-, and 9-hydroxyphenanthrene (OH-PHE), 1-hydroxypyrene (1-OH-PYR) and the sum of 1,6- and 1,8-dihydroxypyrene (Di-OH-PYR).

Spot urine samples of smoking participants were collected from each subject at the beginning of the experiment, after 3 weeks of the treatment period with antioxidant, after additional 3 weeks during which a placebo was given (except for blueberry), and finally after additional 3 weeks considered as a wash-out period. All samples were frozen at -20°C. After shipment to BIU on dry ice the samples were stored at -20°C until used for analysis. To allow the determination of urinary biomarkers of aromatic amines urine samples of non-smoking study participants were taken in the presence of *tert.*-butylamine which was used as required intercept-reactant (*cf* WP8). The urine sampling was modified during the course of the research program. In addition, obstacles concerning the ability of the participants to produce a urine sample also reduced the number of samples occasionally. The final set of samples analysed and validated here is in accordance with the scheme presented above.

Cotinine is a longer-lived metabolite of nicotine and considered as biomarker of tobacco smoke exposure. Its urinary concentration was used to characterize self-reported smoking status indicated by the study participants at Stockholm University in their questionnaires. Cotinine was determined in each urine sample according to a modified procedure using a GC-MS method.

The relationship between the self-reported number of smoked cigarettes per day (CPD) and the corresponding cotinine values determined in the spot-urine samples. No clear relationship could be observed in the investigated smokers. Published data indicate that there is a good linear correlation between urinary cotinine levels and the number of smoked CPD. Several reasons could possibly explain the limited correlation including (i) the self-reported numbers of CPD are incorrect and/or (ii) the smoking habits of the attracted Swedish smokers differs from those of previous studies with respect to number of puffs, puff volume, and puff duration per cigarette and/or (iii) the use of filter tips or filter cigarettes reducing the uptake of tar and nicotine. These issues was not been covered by the questionnaire. In a study performed in the general population by the German Federal Environment Agency, a GM value of cotinine were 0.063 mg per g creatinine and 0.003 mg per g creatinine has been reported for smokers and non-smokers, respectively. It is clear that some detected urinary values for cotinine in the Swedish smokers are in the range observed for German non-smokers.

The analysis of the urinary PAH phenol metabolites (in total 8 phenols of pyrene and phenanthrene) has been performed according to previously established methods using an isotope dilution methodology. All data have been normalized to volume and urinary creatinine level.

To allow the determination of urinary biomarkers of aromatic amines in the Swedish smoker study spot urine samples of the smokers were in the presence of *tert.*-butylamine (stock solution of 1 mg/mL iso-propanol) which was used as required intercept-reactant and frozen at -20°C. After shipment to BIU on dry ice the samples were stored at -20°C until used for analysis. The following aromatic amines have been determined in urine samples of the Swedish smoker study participants as biomarkers of arylamine exposure: *o*-toluidine, 1- and 2-aminonaphthalene (1- and 2-ANa), and 2-, 3-, and 4-aminobiphenyl (2-, 3-, and 4-ABi). The low level determination of arylamines in urine requires a volume of about 50 mL. For some smokers the overall provided volume of urine did not allow the determination of arylamines at each time point.

The determined value of 3-HPMA at BIU in a smokers urine of 1.700 µg/L is in good agreement with the median (1.095 µg/L, N=35) for smokers. Urine analysis in the smoker group the antioxidant chlorophyllin (CHL) has been given orally in doses of 200 mg per day for three weeks, followed by a placebo period and a final wash-out period. Urine samples of two participants became available and have been analysed for 3-hydroxypropylmercapturic acid (3-HPMA), the phenolic PAH metabolites and the aromatic amines. In the following selected data for 3-HPMA, the sum of 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene (sum OH-PHE) and the three aromatic amines known to be human carcinogens including *o*-toluidine, 2-aminonaphthalene (2-ANa) and 4-aminobiphenyl (4-ABi) are discussed. The results of the

urinary 3-hydroxypropylmercapturic acid (3-HPMA) determinations from the experimental scheme with CHL show a great variation (320-1500 µg/L), but as expected are higher than those in non-smokers. For 3-HPMA in German non-smokers median values in a range of 150-170 µg/L have been reported. The results of the experiment with CHL suggest that CHL cannot act in smokers by direct complexation with PAH if their exposure occurs by inhalation and not by co-ingested with food.

In an environmental survey performed in the general population by the German Federal Environment Agency a 1-OH-PYR median value of 0.25 µg/L and 0.1 µg/L has been reported for smokers and non-smokers, respectively. In another study from the GFEA a sum-OH-PHE median value of 0.52 µg/L has been reported for non-smokers. The values observed in Swedish smokers are in line with these reported results. Human exposure to arylamines is known to occur from occupational settings, from food and other environmental sources and in particular from tobacco smoke. The results of the experiment with chlorophyllin a decrease of the levels of o-toluidine, 2-aminonaphthalene (2-ANa) and 4-aminobiphenyl (4-ABi) could be detected. In contrast, both 2-ANa and 4-ABi excretion is increased after the 3 weeks treatment with CHL. It is of interest to note that the levels of most of the arylamines were higher after the wash-out phase compared to those before.

In smokers *o*-toluidine is excreted in significantly higher amounts than in non-smokers. Urinary arylamine excretion in smokers was associated with the extent of smoking as assessed by daily cigarette consumption and cotinine in saliva. All non-smokers investigated had quantifiable amounts of o-toluidine, 2-ANa, and 4-ABi in their urine, confirming that other environmental sources of exposure to these compounds also occur.

In the smoker group with the antioxidant blueberry concentrate (BB), orally doses of 200 mg per day for three weeks were tested. Urine samples have been analysed for 3-hydroxypropylmercapturic acid (3-HPMA) and the phenolic PAH metabolites. The aromatic amines have only been analysed in urines of 2 participants due to limited urine amounts from one participant. In the following selected data for 3-HPMA, the sum of 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene (sum OH-PHE) and the three aromatic amines known to be human carcinogens including *o*-toluidine, 2-ANa and 4-ABi. The results of the urinary 3-HPMA determinations from the experimental scheme with blueberry (BB) concentrate the levels of 3-HPMa were lower after the wash-out phase than before. In contrast to the observation for 3-HPMA the levels for 1-OH-PYR and the sum OH-PHE are unchanged or are increased after the wash-out period. The values for both 1-OH-PYR and the sum OH-PHE are in the range expected for smokers.

In the smoker group with the antioxidant astaxanthin orally in doses of 8 mg per day for three weeks has been given, followed by the placebo period and the final wash-out period. Due to limited amounts of urine, urinary arylamines have been analysed in 2 participants only. All four subjects have 3-HPMA levels before the experiment (800 – 2600 µg/L), which are clearly levels indicative for smokers. The results of the urinary 1-OH-PYR level varied, both increased or decreased similar to the result for 3-HPMA. In comparison to other Swedish smokers in this study both subject had a relative low urinary level of arylamines.

In the smoker group with the antioxidant lycopene orally (via Tomato juice) doses of 24 mg per day for three weeks has been given, followed by the placebo period and the final wash-out period. The urinary 3-HPMA level is reduced after 3 weeks intake of lycopene in 3, whereas an increase is observed for 2 subjects. Lycopene was given to 5 smokers and the results of the determined urinary 1-OH-PYR and the sum OH-PYR showed remarkable similarities to those of the 3-HPMA. Taken together the obtained PAH biomarker data in the lycopene experiment did not allow a conclusive explanation considering lycopene to mediate phase 1 enzyme inhibition and phase 2 enzyme induction which are thought to be the major underlying chemoprotective mechanisms of lycopene against PAH-induced toxicity. The results of the urinary arylamine levels was that lycopene treatment reduced the levels of all three arylamines in three of the smokers but had the opposite effect in the two others.

In the smoker group with the antioxidant *Gentiana lutea* (GL) root powder orally in doses of 800 mg has been given per day for three weeks, followed by the placebo period and the final

wash-out period. Taken together, the data on urinary metabolites could not clearly concluded if GL root powder has an influence on the excretion of the phenolic PAH biomarkers in urine. Taken all data together, the obtained results for the urinary arylamines in this smoker group are to limited to conclude any clear effect for the treatment with the antioxidant GL root powder. Further investigations are required to shed more light on the effects of GL root powder on the toxicokinetics of arylamines in vivo.

Analysis of biomarkers for diabetes. In this part of the study, the plasma of 19 people who were smoking 5-20 cigarettes per day were analysed. Blood was collected three times, at the start of the study, at the end of the antioxidant treatment and after a wash out period. The effects of the peroral intake of blueberry, chlorophyllin, astaxanthine, lycopene and gentiana were analyzed.

IGFBP-1 is a marker of insulin production and insulin resistance and determines the level of free IGF-I. Low IGFBP-1 (< 20 µg/L) is a sign of insulin resistance and gives a high level of free IGF-I. High total IGF-I is associated with higher risk for cancer. The results show that 100% of participants smoking 19 cigarettes per day have low IGFBP-1, leading to increased free IGF-I. At the start of the study 50% of those who are smoking <7 cigarettes per day have low IGFBP-1. All individuals smoking more than 15 cigarettes per day have high IGF-I (corrected for age). Of those smoking more than 7 cigarettes per day 84% had low IGFBP-1.

Astaxanthine consumption had no effect on IGFBP-1 or IGF-I. Lycopene had no effect on IGF-I but IGFBP-1 is decreased in 3 out of 5 persons. Blueberry is decreasing total IGF-I in 4 out of 5 persons without effecting IGFBP-1. Chlorophyllin decreased total IGF-I in 3 out of 6 persons without having larger effect of IGFBP-1. Gentiana was lowering total IGF-I in all cases and is also decreasing IGFBP-1 in 3 out of 5 persons.

It appears from the experiments described above that 84% of those smoking more than 7 cigarettes/day have signs of insulin resistance and a high level of free IGF-I, which increases the risk for type 2 diabetes, arterial and heart diseases and cancer. As a protecting agent astaxanthine has no effect while the others have some positive effects. The best protective substance appears to be blueberry.

Interpretation of all data generated in the intervention studies on smokers. The overall aim of this work package was to study 5 selected dietary supplements on their protective effects on chromosomal damage in smoking individuals. The choice of supplement was according to step-wise strategy for the project. The first step was to select at least 11 the most potentially protective agents based on literature data. The second step was to test these in laboratory tests using in vitro technology. The best outcome in terms of protection against carcinogens that has been identified for exposure in humans was further tested in animal test. A selection of 5 protective agents from animal results was the option for studies in this work package.

The aim and overall objective in this study on humans was to take advantage of expected elevated levels of chromosomal damage due to the effect of smoking. In this study, we also took advantage to investigate the influence of life-style and dietary factors indicated in questionnaires, by clinical analysis of biomarkers for health, by analysis of biomarkers for dose in blood and urine samples and a testing program for health status.

The biomarkers for each individual in the present work package (WP9) can be categorized in three groups; biomarkers of health status/life style, biomarkers of dose and biomarkers of effect. The results are achieved and summarized here by partners SU, KI and BIU on the basis on the findings presented in the individual tasks 1-7 of this work package.

Biomarkers of health included information on gender, age, weight, length, BMI, blood pressure, clinical analysis of B-hemoglobin, CRP, calcium, glycose, albumin, creatinine, ALAT, GT, fP-triglyceride, cholesterol, HDL-cholesterol, LDL-cholesterol, fP-LDL/HDL-cholesterol, S-TSH, S-cobalamin, fS-folate, iron, vitamin C, D and E, and co-enzyme Q10, insomnia, fatigue, stress, illness, upset stomach or any other symptoms, total health test value including oxygen uptake and physical stress test.

Biomarkers of life-style included information on exercise per week, vegetarian, food intake such as fat, proteins, carbohydrates, fibres and fruit and vegetables, total calorie, intake of vitamin C and D or any other vitamin supplements, iron, folate, intake of liquor, wine and beer

per week. Smoking habits, living with a smoker, intake of smokeless tobacco, current work, intake of pain killers, mode regulators, medicine for cancer, psoriasis, illness or any other medication, X-ray diagnostic.

Biomarkers of dose for smoking and food exposure included analysis of protein adducts in blood for acrylamide, glycidamide, ethylene oxide, acrylonitrile and several others in an adductomics approach. Another biomarker monitored was the oxidative stress marker 8-OxodG. From urine the metabolites aniline, cotinine, o-toluidine, m-toluidine, p-toluidine, 1-aminonaphthaline, 2-aminonaphthaline, 2-aminobiphenyl, 3-aminobiphenyl, 4-aminobiphenyl, 1-aminopyrene, 1-hydroxypyrene, dihydroxypyrene, 1-OH-phenanthrene, 2-OH-phenanthrene, 3-OH-phenanthrene, 4-OH-phenanthrene, 9-OH-phenanthrene, 3-HPMA.

Biomarkers of effect involves analysis of chromosomal damage using the frequency of peripheral micronucleated erythrocytes (fMN) corresponding to a short term effect on the bone marrow stem cells. The erythropoiesis in mammals takes place in the bone marrow. After stem cell divisions, the erythroblasts expel their nucleus and migrate out in the peripheral blood stream. If chromosomal damage occur or chromosomes do not migrate to daughter cells a micronucleus is formed which is not expelled. Young polychromatic erythrocytes can be recognized in the blood stream for approximately 24 hours before they maturate to normochromatic. Micronucleated cells are selected in the spleen why they can only be seen in the peripheral blood for a few days. The advantage of this is that any affect in the bone marrow can be detected within a few days only without a background frequency induced by historical exposures. Blood samples from 28 individuals were analysed for MN-Trf-Ret. We used the FACS-based assay for MN in Trf-Ret described elsewhere. On average, 1.5-2 ml of blood was needed for the analysis.

All together 28 voluntary cigarette smokers were recruited among 60 000 students and 5 000 employees at Stockholm University Campus. The volunteers were allocated randomly to the different supplements to be investigated and in the order they applied to take part in the study. At least 5 cigarettes per day were required to take part in the study but the strategy was to find volunteers smoking as much as possible. The problem to perform this type of study in Sweden is that many people stop or reduce their smoking habits. The smoking status for each individual were documented in questionnaires and by analysis of cotinine in the urine. Cotinine has for a long time being used as a trustful biomarker for smoking habits.

From the results of cotinine analysis, it is clear that the levels of cotinine is not in agreement with what was self-reported for smoking habits by the participants. However, published data indicate that there is a good linear correlation between urinary cotinine levels and the number of smoked CPD. Several reasons could possibly explain the limited correlation including (i) the self-reported numbers of CPD are incorrect and/or (ii) the smoking habits of the attracted Swedish smokers differs from those of previous studies with respect to number of puffs, puff volume, and puff duration per cigarette and/or (iii) the use of filter tips or filter cigarettes reducing the uptake of tar and nicotine. These issues was not been covered by the questionnaire. Since antioxidant/protective agents could act on any life-style factor, even participant with low cotinine levels were included in the analysis of their effect on biomarkers. The reason was that not only smoking were expected to elevate the level of the different biomarkers, such as different diet and other life-style factors. This was in fact what could be demonstrated when overall results were analysed.

Initially blood samples from the smoking participants were analysed for all biomarkers including chromosomal damage. From the beginning, 28 volunteers were recruited. For different reasons, 20 or 21 were included in the analysis depending of the time point they wanted to finish their participation. A set of 19 non-smoking volunteers were recruited and analysed in comparison. Since there was a weak correlation between self-reported smoking habits and cotinine levels, this was in agreement with the non-significant relation between the number of self-reported number of cigarettes per day and the level of chromosomal damage (Fig. 3a). This relation was not improved comparing the MN levels to the cotinine levels suggesting the importance of other life-style factors involved in the chromosomal damage level of each individual. However, as a group, smoking was indicated as a significant factor

compared to non-smoking individuals (Fig. 3c) supporting that smoking is an important life-style factor.
Therefore, any individual effect of a tested dietary supplement is expected to come out on the effect of life-style factors all together effecting the individual rather than on the effect of smoking alone.

When comparison were performed between cotinine levels and the different biomarkers covering smoking habits, significant and satisfying correlation were found. Although it was found that smoking habits could be detected as a group, no relation between individual or total levels of protein adducts and fMN was found.

Analysis of biomarkers, e.g., 17 markers for smoking and diet, were performed in urine samples. A significant relation between increase in fMN and increase in both 1-OH-pyrene and 3-OH-phenanthrene were found suggesting exposure of genotoxic agents in cigarette smoke and urban air. A negative correlation between the three toluidines were indicated. Taken all results together concerning biomarkers for smoking, there was clear evidence for exposure of smoking, although it was difficult to show this clearly on individual basis.

The statistical analysis all data achieved at the first sampling was multivariate. We used recursive portioning of life-style versus fMN (chromosomal damage) as a marker for genotoxic risk. Life-style exposure is covered by several hundred biomarkers in this study. Protein adducts cover genotoxic exposure from heated food, smoking and urban air. Urinary metabolites cover food, smoking and some air exposure coming from combustion, e.g., polyaromatic hydrocarbons (PAHs). Also biomarker for health, food and information in questionnaire (included in life-style) were tested for their influence on fMN. Non-significant factors are not discussed in this summary but fully reported in the individual deliverables of this work package. Folate, age have been found by others, smoking years is not a surprise but HDL-cholesterol is an interesting indication of interaction with lipid metabolism, although here found to be rather weak.

The statistical significant results using recursive portioning of life-style versus fMN for 21 smokers suggested that wine consumption is the first factor of importance. For those drinking less than 2 glass of wine per week, smoking years comes out as second important factor and interestingly, beers comes out as protective for those drinking more than 2 glass wine per week. Smoking and wine gives as high as 2.5 times background compared to non-smokers suggesting a synergistic effect between smoking and alcohol, in agreement with what has been well known for a long time for cancer in the mouth cavity.

For 19 non-smokers, medication comes out as the most important factor followed by gender and females giving high values. Individuals with no medication, interestingly, intake of vitamin supplements was protective and also the lowest value in the data set. It was clear that there was an interaction between smoking and drinking, which is well-known from epidemiological studies on cancer in the mouth cavity.

When factors were analysed individually for the 21 smokers, cancer and psoriasis treatment, living with a smoker, X-ray diagnostic, and wine consumption over 2 glass per week were all statistical significant enhancing genotoxic risk. Statistical significant protection were shown to be vitamin intake and being a vegetarian.

The intervention was performed in three periods, a 3 weeks period involving the intake of supplement started and ended by a sampling of blood and urine. This period is followed by a placebo period with intake of the same type of capsule and ended by another 3 weeks period for wash out period, also ended by sampling. The sampling for the 8-OxodG tolerance was performed twice on the sampling day, before and 1 hour after ergonomic cycling for 20 minutes at an intensity of 80% of theoretical pulse speed. The time of intervention was 9 weeks with 3 different intervention conditions. After delay, the procedure for intervention with 24 volunteers starting 28 interventions of which 25 were completed successfully.

The first supplement was 5 volunteers having freeze-dried blueberries, organic certified and harvested in Scandinavia. The intake was 4 g/day which correspond to 28 g fresh blueberries containing about 200 mg anthocyanines. The effect of blueberry on MN levels was clearly significant although only volunteers with elevated background levels of MN are effected.by the

antioxidant. The result is of interest because it suggest that individuals with “normal” levels of MN have sufficient protection against agents that induce chromosomal damage. After washout, none of the individuals indicated an effect of the antioxidant. The placebo was not included in this part of the study concerning blueberry because of its significant taste and smell. The effect was not seen after the wash out period, indicated a rather short half-life of the active substance(s). It should be pointed out that 5 individuals is very few and the observation should be regarded as very preliminary. More individuals are needed for conformation and a more solid evaluation. The protective effect on 8-OxodG, protein adducts or urinary metabolites due to the intervention of supplements could not be detected. Since these biomarker are detecting doses levels of different types of exposure, it probably requires a more long term exposure to be detected.

The second supplement was chlorophylline, an approved food colouring agent (E141). The intake was 200 mg/day. The dye is water soluble and with a similar structure as chlorophyll. It can inhibit binding of reactive molecules to cellular macromolecules by sequestering mechanism. 5 volunteers used to investigate this supplement. The effect of chlorophylline on MN levels was not statistical significant although all values lies in the range for detection according by the findings from Blueberry. It suggests that the variation was too large and more individual were needed to detect any effect of this supplement. As noted for blueberry, the results are very preliminary. A protective effect on 8-OxodG, protein adducts or urinary metabolites due to the intervention of supplements could not be detected. Since these biomarkers are detecting doses levels of different types of exposure, it probably requires a more long term exposure to be detected.

The third antioxidant was astaxanthine, a carotenoid produced by algae. It is produced as an oil emulsion when used as dietary supplement. The dose to 6 volunteers was 8 mg/day. The effect of astaxanthine was statistical significant and similar to that of blueberry, suggesting that only individuals with enhanced levels of MN were beneficial for an effect of antioxidant supplementation. Opposite to chlorophylline, the correlation between the effect of astaxanthine and background MN levels were statistical significant. Note that one of the volunteers did not participate throughout the study. A significant protection was found even after the wash out period indicating that this compound was very stable in vivo. As noted for blueberry, the results of intervention with astaxanthine must be regarded as very preliminary. The protective effect on 8-OxodG, protein adducts or urinary metabolites due to the intervention of supplements could not be detected. Since these biomarkers are detecting doses levels of different types of exposure, it probably requires a more long term exposure to be detected.

Capsules containing lycopene extract from tomatoes was given as the fourth antioxidant to 5 volunteers. It provides the synergy of the natural tomato lycopene, phytoene, phytofluene, beta-carotene, phytosterols and vitamin E. One capsule correspond to approximately 15 mg lycopene. The intervention with Lycopene lacks the possibility for detection of a protective effect. These to interventions suffered from the “normal” background levels of the volunteers, why a reduction was not expected to be detected. The protective effect on 8-OxodG, protein adducts or urinary metabolites due to the intervention of supplements could not be detected. Since these biomarkers are detecting doses levels of different types of exposure, it probably requires a more long term exposure to be detected.

Last antioxidant was an endemic herb from central and southern Europe, Gentianella lutea. It is traditionally used as a flavour used in beverages in Serbia. The preparation was ingested by 6 volunteers taking one capsule/day containing 400 mg/capsule. The intervention with both Gentianella lack the possibility for detection of a protective effect against MN. These to interventions suffered from the “normal” background levels of the volunteers, why a reduction was not expected to be detected. The intervention with both lycopene and Gentiana lack the possibility to detect a protective effect. These to interventions suffered from the “normal” background levels of the volunteers. The protective effect on 8-OxodG, protein adducts or urinary metabolites due to the intervention of supplements could not be detected. Since these biomarkers are detecting doses levels of different types of exposure, it probably requires a more long term exposure to be detected. The same argument goes for detection of a

difference in protein adducts during a intervention time of only 3 weeks because of the life time of peripheral red blood cells are around18 weeks.
The overall results from intervention with smokers clearly show that the effect on MN levels of the antioxidants can only be detected when the individuals have elevated background levels. The level does not necessary be the consequence of smoking, supported by the fact that there were no correlation between number of self-reported cigarettes per day and MN levels. The results indicate that the effect of the antioxidant stayed for approximately 3 weeks, where after it started to fade off during the three placebo weeks and further during the washout period. The biomarkers for diabetes were to some extent successful, although the number of individuals was limited.

Modelling of data from smokers and non-smokers for genotoxic risk. The multivariate data analysis and the results from recursive partitioning can be used for modelling of individual health prediction concerning genotoxic risk. Various evidences indicate the importance of environmental factors as contributors to human health risks. In this context environment has to be considered in a broad sense and cover the totality of environmental exposures over a life-time (so called exposome) including lifestyle factors, environmental chemicals, occupation, nutrition, but also include chemicals produced in the body by endogenous natural processes such as oxidative stress, lipid peroxidation, etc.. It has been shown that different dietary factors contribute to both enhanced and reduced risk for cancer (WCRF/AICR, 2007). There are, for instance, convincing evidence for increased risk of cancer from alcohol and from red meat. Non-starchy vegetables and fruits together with physical activity have been identified as the most important factors for a reduction of cancer risk. It can be assumed that the balance in the composition of the diet is related to the risk and that the combined dietary factors interact with other factors, to make up the individual's cancer risk.

The overall results generated in this project can be used for modelling of such factors of interest for risk for cancer. The developed sensitive biomarkers for assessment of lifestyle in a broad sense causing genotoxicity, oxidative stress, etc., can be used for modelling of genotoxic risk for genotoxic carcinogens in humans. Here we provide an example coming from the results achieved in this work package.

The results from the identified lifestyle factors of statistical significance have been object for of model to predict health status. Based on this model, a tool was constructed to identify individuals at risk for enhanced DNA damage levels and consequently possible enhanced risk for disease. From WP9 we used 42 individuals for prediction of their enhanced risk for disease by testing the risk model transformed in a short questionnaire to answer lifestyle factors of importance. This strategy was to construct a "learning set" for prediction of genoxic risk. The score for each life-style factor was related to the strength in the multivariate analysis. The scores for the factors were; smoking -4, taking medicine -2, having cancer or psoriasis treatments -1.5, drinking more than 2 glass of wine per week -1.5, sharing home with a smoker -1, undergone diagnostic X-ray -1, taking vitamin supplementation +2, being a vegetarian +3. From the life-style score we constructed a semi-quantitative scale for risk for genetic damage. Those individuals that had scores below –4 ("Enhanced"), as compared to those with scores between -4 to 0 ("Normal") or those above 0 ("Low"). The score significantly correlated with the level of DNA damage. The data was compared to the actual MN levels found in this "learning set". A highly significant correlation was found suggesting that the most important factors for genotoxic risk was found. The model will be refined and further testing will be performed in upcoming studies supported by others in which a "testing set". New volunteers will be asked to answer the questions in the life-style protocol and thereafter, for validation of the model, be tested for genoxicity using the MN analysis of a blood sample. The impressive task is that this prediction method when it comes to use will be non-invasive which means that it can be used free of charge by a large number of individuals without visiting a clinic.

Concluding remarks: The results achieved in this work package have given both knowledge and experience in the assessment of genotoxic risk by life-style and dietary factors. Since genotoxicity is an important first step in the cancer process, the finding has also impact in cancer risk assessment. The biomarkers used here were shown to be sensitive enough to

detect effect from life-style above background levels. The biomarkers were also shown to be fast and cost effective. Several life-style factors were identified and quantified relative overall genotoxic risk. A model could be constructed which was shown to be significant although the number of individuals in the study was limited. The results will pave the way towards a more narrow identification of life-style factors in order to approve the quantification of genotoxic risk.

Work package 10 (WP10) concerned the protective effects in human exposed to carcinogens in vapors from heated cooking oils. This work package has been conducted by the Indian partners. It will be reported by the Indian participants to DBB, India.

Work package 11 (WP11) concerned the impact of protective agents on cultures of vascular smooth muscle cell (VSMC) atherogenesis. This work package has been conducted by the Indian partners. It will be reported by the Indian participants to DBB, India.

Work package 12 (WP12) concerned the antiglycating and aldose reductase inhibition as indicators of protection against diabetic ocular complications. This work package has been conducted by the Indian partners. It will be reported by the Indian participants to DBB, India.

Work package 13 (WP13) – concerned *in vitro* studies on the protective action of selected putative protective agents with respect to oxidative stress in human cells subjected to high glucose concentrations and hypoxia.

Human primary fibroblasts were cultured in a high concentration of glucose ±hypoxia in order to induce oxidative stress, a condition that occurs in diabetes. Proliferation of human fibroblast (measured as tritiated thymidine incorporation) is inhibited by high glucose. Coenzyme Q10 (CoQ), and carnosine were found to counteract this inhibition, while ellagic acid, resveratrol, theaphenone E, astaxanthine, and the *Gentiana* extract had no effect. Hypoxia combined with high glucose is also inhibitory, and in this case, in addition to CoQ, the *Gentiana* extract and astaxanthin restored normal proliferation. Elagic acid, resveratrol and teaphenone E were not effective under these conditions. High glucose levels increase PLPase-2 activity (increased amount of substrate for inflammatory prostaglandins), an elevation that was significantly suppressed in the presence of CoQ.

Kidney disease is a common sequel of diabetes. Kidney mitochondrial respiration and the rate of ATP synthesis was quantified. Increased respiratory rates were induced by CoQ, and carnosine, resveratrol and astaxanthine were somewhat less effective,, whereas and teaphone E, although the level of response varied greatly. CoQ, carnosine, followed by resveratrol and astaxanthin displayed a significant impact, and to a moderate extent teaphenone E and the *Gentiana* extract. *Astagalus* extract and proanthocyanidins (from pine bark containing the oligomeric form) were obtained from health shops and their concentrations were estimated by UV-absorption and HPLC-chromatography. Their effects on parameters mentioned above were moderately positive.

Molecular biological analysis of RNA extracts from fibroblasts and from endothelial and smooth muscle cells were systematically analyzed concerning some of the target genes expressed under high glucose and hypoxia treatment. The mRNA measured with RT-PRC were HIF1α, GLUT-1, VEGF, PDGFB, PDGFBR, PGC1α, TGFα, IGF-I, EGF, angiopoetin-2 and IGFBP-1. Experiments with rutin and gingerol were conducted, as well as for an *Astagalus* extract and proanthocyanidins (from pine bark containing the oligomeric form) obtained from health shops. Their actual concentrations were estimated by UV-absorption and HPLC-chromatography. The effects on the chosen parameters were weakly positive. For compounds giving a positive response, Western blot was also employed. Induction of target genes occurred in various extents and the most striking induction was obtained in tissue culture cells after incubation with CoQ, resveratrol, carnosine and astaxanthin, while induction by the other substances tested was moderate.

Dbb mice are used for studies of protection against diabetes, nephropathy and wound healing *in vivo.* CoQ, carnosine and astaxanthin were selected for 4 weeks` treatment in

diabetic db/db mice and GK-rats aged 8-12 months. Following treatment a glucose tolerance test was performed (blood glucose, insulin). It was reported that the average decrease in blood glucose level was 18 % for CoQ, 12 % for carnosine and the decrease for astaxanthin was statistically not significant. The CoQ content of the plasma and the liver was increased by 65 and 18 % after CoQ administration, by 21 and 14 % after carnosine treatment and 14 and 10 % after astaxanthin diet, respectively. IGF-I values in the plasma increased by 36, 38 and 19 % and in the liver by 22, 26 and 11 % after employing the 3 types of diets

Treatment with dietary CoQ for two weeks prevented glutamate stimulated oxygen consumption (i.e. mitochondrial uncoupling). The beneficial effect of CoQ on mitochondrial function in diabetic kidneys might include both decreased UCP-2 activation but also reduced UCP-2 protein levels. Carnosine treatment for 4 weeks in db/db mice substantially decreased renal carnosinase activity in diabetic mice and close to normal levels was attained. Renal anserine concentrations increased significantly in treated db/db mice while carnosine concentrations remained unaltered. Further, carnosine treatment halved proteinuria and reduced vascular permeability to one-fifth.

During the *in vitro* studies, applying food additives to tissue culture systems, it was concluded that several tested substances had positive effects on various metabolic functions. The two substances that in general gave the most advantageous responses were carnosine and coenzyme Q (CoQ). These two compounds were therefore selected for *in vivo* studies, also including the diabetic models db/db mice and GK-rats.

In diabetes model systems and in patients mutations are often found in the carnosinase gene leading to high carnosinase activity. The decreased carnosine and anserine concentrations have serious deleterious effects in several organs with major effect on the kidney. In the kidneys of the diabetic mice carnosinase activity was greatly increased and anserine concentration was decreased. Upon treatment of mice with carnosine the carnosinase activity returned to the low control level and anserine concentration is highly elevated in the renal tissue. Using Evans blue it was also found that the pathological vascular permeability in the db/db mice after 8 weeks treatment with carnosine returned the control level.

Diabetes involves a number of severe late complications. One of them is impaired wound healing, causing working inability and great a cost for the society. When experimental wounds in db/db mice were treated with carnosine, the rate of healing was improved as early as the second day of treatment. Serum analysis at the end of the treatment showed an increase in serum carnosine level. The tissue at the wound area was also investigated and the analyses demonstrated an increased expression of growth factor and cytokine genes involved in wound healing. Carnosine also increased the cellular viability and migration efficiency.

Increased oxygen consumption results in kidney tissue hypoxia, which contributes to the development of diabetic nephropathy. Untreated db/db mice display mitochondrial uncoupling, glomerular hyperfiltration, increased proteinuria and mitochondrial fragmentation. These alterations could be prevented or reduced by CoQ treatment. It was found that the glutamate-stimulated oxygen consumption is greatly increased in isolated kidney mitochondria of the diabetic mice and the oxygen consumption is greatly reduced when the mice are treated with CoQ during 7 weeks. The conclusion is that the oxidative stress-mediated activation of UCP-2 is the reason of mitochondrial uncoupling and this is effectively counteracted by the antioxidant CoQ.

Db/db mice were placed on diets containing CoQ, carnosine and astaxanthin for 6 months, and tests were performed to estimate the extent of neuropathy. The db/db mice exhibited peripheral neuropathy characterized by morphological, pathophysiological and neurochemical changes in dorsal root ganglion neurons. These mice develop tactile and thermal hypoalgesia. Using Western blot, Bax were found to be significantly higher, while p-Akt and p-Erk were significantly lower in diabetic primary sensory neurons. There was also a considerable reduction of phospholipase C β3, an important intracellular signalling molecule. The reduction was greatly inhibited by coenzyme Q (CoQ) treatment of the mice, the reduction with carnosine and astaxanthin was also considerable, but 20-30% less than with CoQ. To a

similar extent these treatments inhibited the reduction of the sciatic nerve conduction velocity. All three supplements improved tactile hypoalgesia. Thermal response thresholds decreased in response to treatment with the three supplements. Body weight and blood glucose levels were not changed after treatment with these compounds.

Diabetic peripheral neuropathy is the most common complication in both type 1 and type 2 diabetes. It was found that diabetic mice at 8 months of age exhibited loss of sensation, hypoalgesia, decreased mechanical hyperalgesia, cold allodynia and decrease sciatic nerve conduction velocity. Daily CoQ treatment during 6 months improved these pathological conditions to a large extent. The histochemical study showed a significant reduction in the proportion of medium-sized neuron profiles. The stereological analysis displayed a 33% neuronal cell loss in diabetic mice. The neuronal loss was only 13% after CoQ treatment.

Work package 14 (WP14) concerned investigation of functional foods/constituents in humans with respect to protection against diabetes and CVD. The design of the study was to investigate the preventive effect of 3 months´ treatment with 2 putative protective agents on the development of diabetes and its complications. Recruitment of 40 prediabetic and 40 type 2 diabetic patients without significant complications; 20 males and 20 females in each group.

A geographically defined population consisting of 141 subjects with type 2 diabetes, 59% males and 41% females, with a diabetes duration of 7±5.5 years, was identified for further investigation. The subjects were interviewed, their medical records assessed, and informed consent obtained.

The investigations so far performed included serum concentrations of coenzyme Q10 and vitamin E, hydrogen peroxide generation status. Reactive oxygen species (ROS) was determined by free oxygen radicals testing (FORT). A large number of relevant urinary and serum biomarkers, and waist index were determined using with established methods. Diabetic peripheral sensory neuropathy was assessed, and subjects with excessive consumption of alcohol, or treatment with B12, as well as those with a history of myocardial infarction and renal failure were excluded from the study.

47 prediabetic individuals have been recruited, who have impaired glucose tolerance without diabetes according to the performed oral glucose tolerance test. Additionally, insulin and proinsulin were also measured. The subjects, who completed questionnaires, were subjected to clinical examination, blood and urine sampling were taken and the medical records were reviewed. Patients with a history of chronic heart failure, liver diseases, renal failure or rheumatoid diseases were excluded from the group. Informed consent was obtained.

67 healthy individuals have been selected and subjected to physical examination including blood pressure, heart, lung and nerve function. A glucose tolerance test was performed, and routine blood and urine analysis conducted to verify normal values. Subjects with signs of chronic disease or malfunction of unclear origin were excluded. In addition blood samples from a previous study from Serbia (around Belgrade) were investigated. In this population (100 female with an age-range of 19-59) the consumption of smoked food was three times higher than the female population around Stockholm (100 females). Concerning the nutritional habits the only difference was a higher consumption of fish and a slightly higher (20 %) consumption of all vegetables and fruits of the Serbian cohorts. Blood glucose values were similar and in the normal range in both populations. No significant differences could be observed in serum cholesterol (both LDL and HDL) and triglyceride levels. Coenzyme Q (CoQ) levels of the Swedish women show a narrow range, the mean value around 1 nmol/ml. In the Serbian cohort the range was 1.5 to 3.5 nmol/ml (mean value 1.95). The vitamin E levels in the serum of Swedish woman had a concentration of 20-40 nmol/ml (mean value 23) while for the Serbian cohort it ranged between 40 and 80 nmol/ml (mean value 49). The vitamin E/CoQ ratio was similar, 24.7 for Serbia and 23.5 for Sweden. The Swedish females had a majority of SD-score values between -0.5 and +0.5 (mean 0.070) while in the Serbian population the values were considerably lower (mean -1.13). In the Swedish population most of the IGFBP-1values were in the range of 20-40 µg/l and about one third of the values were between 40 and 70 µg/l (mean 27). The Serbian group had a low level of IGFBP-1, below 20 µg/l (mean

13). Low IGF-I values are unfavourable since they increase the risk for cancer and arteriosclerosis, however, genetic factors and chronic inflammation can also lead to low values. A low level of IGFBP-1 suggests insulin resistance and is a factor for increased risk for cardiovascular disease and type 2 diabetes.

Tests was performed with biochemical markers for protection against diabetes and CVD. Three groups of persons were recruited, healthy subjects, prediabetic and diabetic patients. The healthy group is defined by fasting glucose below 7.0 mmol/L and oral glucose tolerance test (OGTT) at 120 min below 7.8 mmol/L. Prediabetes is defined by fasting glucose values between 7 and 8 mmol/L and between 7.8 and 11.0 mmol/L when the value at 120 min of the OGTT was performed. Diabetic values are above 8.0 mmol/L as fasting glucose and above 11.0 at 120 min when the OGTT is used. Studies so far have been performed employing coenzyme (CoQ) supplementation and further studies are also initiated using carnosine and blueberry drinks. CoQ was supplemented as 100 mg twice daily which increased the level of this compound in the blood by 2-3 fold. The major part of investigations are performed by the use of oxidized CoQ which after uptake is reduced by a number of enzymatic systems and fully active as antioxidant. We have also started studies supplying the reduced form of this lipid, which is not more efficient in the blood or tissues, however, its uptake is more efficient and double the blood concentration. After 12-weeks of treatment with CoQ it was found that LDL was decreased in both types of diabetes. There was also a decrease of oxidized LDL. The level of HDL remained unchanged. HbA1c, the long-time indicator of the diabetic condition, was improved in type 1 but not in type 2 diabetes. It is possible that in this case a longer time period of treatment is required. A series of cytokines, monocyte adhesion factors and blood proteins were investigated and several of them, such as IGFBP-1 and VCAM, were decreased after treatment of diabetic patients.

The nature and behaviour of NK cells appeared of importance. Supplementation with 100 g CoQ10 twice daily affects NK cell composition and phenotype in diabetic patients. After supplementation, there is a relative increase in the more cytokine producing CD56bright as opposed to the more cytotoxic CD56dim subset. The increased CD11c expression also indicates an increased cytokine production. The upregulated NKG2D expression is associated with NK-cell activation.

Oxidative stress is one the main factors both in development of diabetes and in diabetic complications. We have used several markers for determination of this condition and the results of plasma glutaredoxin activity. Significant up regulation of the activity of this antioxidant protection mechanism in diabetic subject was apparent.

The 12-week treatment with CoQ highly influenced plasma glutaredoxin activity. The high activities in both type 1 and type 2 diabetes were greatly decreased, indicating the lowered levels of free radicals generated during the pathological conditions.

We have started other trials with food supplements. In this case serum thiol concentration was determined as marker of oxidative stress. Subjects consumed blueberry drinks, Coca-cola or fructose drinks. Blueberry drinks were effective in decreasing the blood thiol concentration and is acting as an effective antioxidative agent. Our preliminary results indicate that blueberry is also efficient in decreasing the blood glucose level in diabetic patients.

Work package 15 (WP15) Dissemination of the results have included construction of a web-site, preparation of two newsletter, one in the beginning and one in the end, contributions to international scientific meetings, established contacts and invited commercial establishments for meetings, scientific exchanges between partners and others. Several of the involved scientist have been involved in different public media due to press releases related to the project. The coordinator have been interviewed by governmental organisation concerning the outcome and the health consequences of the results generated in the project. 20 publications by the European partners and 15 by the Indian partners have been accepted in highly ranked journals, as listed below. The publications concerning results generating during the FUNCFOOD project by participant will be a process spanning over several years, since many of the data have been generated at the end of the project. Much of the results will be followed

up by further studies supported by others and consequently lead to additional scientific publications.

Publications related to the project by the European participants:

- Harms-Ringdahl M, Jenssen D, Haghdoost S.,Tomato juice intake suppressed serum concentration of 8-oxodG after extensive physical activity., Nutr J. 2012 May 2;11:29.
- Peters V, Schmitt CP, Zschocke J, Gross ML, Brismar K, Forsberg E. Carnosine treatment largely prevents alterations of renal carnosine metabolism in diabetic mice. Amino Acids. 2012 Jun;42(6):2411-6.
- Persson MF, Franzén S, Catrina SB, Dallner G, Hansell P, Brismar K, Palm F. Coenzyme Q10 prevents GDP-sensitive mitochondrial uncoupling, glomerular hyperfiltration and proteinuria in kidneys from db/db mice as a model of type 2 diabetes. Diabetologia. 2012 May;55(5):1535-43.
- Ansurudeen I, Sunkari VG, Grünler J, Peters V, Schmitt CP, Catrina SB, Brismar K, Forsberg EA. Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes. Amino Acids. 2012 Jul;43(1):127-34.
- Shi TJ, Zhang MD, Zeberg H, Nilsson J, Grünler J, Liu SX, Xiang Q, Persson J, Fried KJ, Catrina SB, Watanabe M, Arhem P, Brismar K, Hökfelt TG. Coenzyme Q10 prevents peripheral neuropathy and attenuates neuron loss in the db-/db- mouse, a type 2 diabetes model. Proc Natl Acad Sci U S A. 2013, 110(2):690-5.
- Berni A, Grossi MR, Pepe G, FILIPPI S., Muthukumar S, Papeschi C, Natarajan AT, Palitti F. (2012). Protective effect of ellagic acid (EA) on micronucleus formation induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in mammalian cells, in in vitro assays and in vivo. Mutation research. Fundamental and molecular mechanisms of mutagenesis, vol. 746; p. 60-65, ISSN: 1386-1964.
- Branislav Nastasijevic Tamara Lazarevic-Pasti, Suzana Dimitrijevic-Brankovic, Igor Pasti, Ana Vujacic, Gordana Joksic, Vesna Vasic, Inhibition of myeloperoxidase and antioxidative activity of Gentiana lutea extracts, Journal of Pharmaceutical and Biomedical Analysis, vol. 66 br. , (2012), str. 191-196
- Grossi MR, Berni A, Pepe G, Filippi S, Meschini R, Papeschi C, Natarajan AT, Palitti F. Evaluation of the effects of ellagic acid (EA) on 7,12-dimethylbenz(α) anthracene (DMBA) induced micronuclei in mammalian cells in vitro and in vivo. Toxicol Lett. 2014 Jan 13;224(2):240-5. doi: 10.1016/j.toxlet.2013.10.012. Epub 2013 Nov
- Gaetano Pepe, Maria Rosaria Grossi Andrea Berni*, Silvia Filippi, Rathina Kumar Shanmugakani,Cristiano Papeschi, Adayapalam T. Natarajan and Fabrizio Palitti (2013) Effect of blueberry extracts (BB) on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced mcronuclei in mammalian cells in vitro and in vivo assays. Mutat Res. 2013 Dec 12;758(1-2):6-11. doi: 10.1016/j.mrgentox.2013.07.012. Epub 2013 Sep 20.
- B. Nastasijević, T. Lazarević Pašti, A. Leskovac, S. Petrović, S. D. Branković, I. Pašti, G. Joksić, V. Vasić, Recent findings about healing properties of *Gentiana lutea* root extracts, 3rd Workshop "Specific Methods on Food Safety and Quality Sept. 27, 2012, Vinca, Belgrade, Serbia, Proceedings OP9.
- V.Vasic, B.Nastasijevic, M.Stoiljkovic, G.Joksic, Influence of grilling meat on PAHs level – experiences of FP7 project FUNCFOOD, 3rd Workshop "Specific Methods on Food Safety and Quality Sept. 27, 2012, Vinca, Belgrade, Serbia, Proceedings OP7.

- B. Nastasijević, M. Stoiljković, N. Vuković, J. Savić, Ž. Todorović, G. Joksić, V. Vasić, Comparison of UPLC/TUV and GC/MS in analysis of PAHs in grilled meat, Euroanalysis-16, Sept. 11-15, 2011, Belgrade, Serbia, FA76.
- B. Nastasijević, Milovan Stoiljković, N. Vuković, Marija Cindrić, Žaklina Todorović, Gordana Joksić, Vesna Vasić, Application of Ultra High Performance Chromatography (UPLC) for analysis of Polycyclic Aromatic Hydrocarbons (PAHs) in grilled and smoked meat, 1st International Conference of Agriculture Food Environment, May 27-28, 2011, Korca, Albania, Book of abstracts, p.217
- Meschini R.,Berni A. Filippi S,Pepe G. ,Grossi MR., Natarajan AT.,Palitti F. Evaluation of the effects of *Gentiana lutea and 6 Gingerol* on by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(α) anthracene (DMBA) induced micronuclei in human hepatoma cells *in vitro* in press. Mutat. Res.
- Brauner H, Lüthje P, Grünler J, Ekberg NR, Dallner G, Brismar K, Brauner A. Markers of innate immune activity in type 1 and type 2 diabetes and the effect of the antioxidant CoQ10 on inflammatory activity, in manuscript.
- Du Y, Zhang H, Montano S, Hegestam J, Ekberg NR, Holmgren, K, Brismar K, Ungerstedt JS. Plasma glutaredoxin activity in healthy subjects and patients with abnormal glucose levels or overt type 2 diabetes, in manuscript.
- Vare, D., Lagerqvist, A., Kotova, N., Jenssen, D. Screening of protective food constituents against genotoxicity in vitro, in manuscript.
- Andrea Berni, Maria Rosaria Grossi, Gaetano Pepe, Adayapalam T. Natarajan, Fabrizio Palitti The effect of Chlorophyllin on genome stability and cytotoxicity in a human hepatoma cell line using the cytokinesis-block micronucleus assay. Atti SIMA 2010, Ab 10, p 21.
- Gaetano Pepe, Maria Rosaria Grossi, Andrea Berni, Silvia Filippi, Muthukumar Sowndarya, Adayapalam T. Natarajan, Cristiano Papeschi, Fabrizio Palitti In vitro and in vivo effect of ellagic acid on MNNG-induced DNA damage. Atti SIMA 201
- Fabrizio Palitti, Andrea Berni, Maria Rosaria Grossi, Gaetano Pepe, Silvia Filippi Sowndarya Muthukumar, Cristiano Papeschi and Adayapalam T. Natarayan Chromosomal damage to assess health benefit of functional foods. 37th Annual conference of EMSI “Molecular basis of human health in response to mutagens and environmental stress. Kiit, February, 24-26th, 2012.
- M.R. Grossi, S. Filippi, A. Berni, G. Pepe, F. Palitti, Il danno cromosomico nella valutazione degli effetti benefici dei cibi funzionali sulla salute umana. Lilt Rimini 26-27 ottobre 2012 “Biotecnologie, salute e nutrizione”.
- Palitti Fabrizio, Andrea Berni, Maria Rosaria Grossi, Gaetano Pepe, Silvia Filippi, Sowndarya Muthukumar, Cristiano Papeschi and Adayapalam T. Natarajan. Chromosomal damage to asses health benefit of functional foods. 11thInt. Conf. on Envir.Mutagens (ICEM), 03 to 08 Nov. 2013 Foz do Iguassu, Brazil

Publications related to the project by the Indian participants:

- Uma Rani P, Rushendhiran K, Raghu G, Avaneesh T, Kumar PU, Reddy GB, Dixit M (2013) Ellagic acid inhibits PDGF-BB induced vascular smooth muscle cell proliferation and prevents atheroma formation in streptozotocin-induced diabetic rats. *J Nutr Biochem* S0955-2863(13)00093-4.
- Kavitha K, Kowshik J, Kranthi Kiran Kishore T, Baba AB, Nagini S. Astaxanthin inhibits NF-□B and Wnt/□-catenin signaling pathways via inactivation of Erk/MAPK and

PI3K/Akt to induce intrinsic apoptosis in a hamster model of oral cancer. *Biochim Biophys Acta GEN* 1830:4433-4444.
- Kesavan R, Potunuru UR, Nastasijević B, T A, Joksić G, Dixit M. (2013) Inhibition of vascular smooth muscle cell proliferation by Gentiana lutea root extracts. *PLoS One*. 2013 8:e61393.
- Anitha P, Vidya Priyadarsini R, Kavitha K, Thiyagarajan P, Nagini S. (2013) Ellagic acid coordinately attenuates Wnt/β-catenin and NF-κB signaling pathways to induce intrinsic apoptosis in an animal model of oral oncogenesis. *Eur J Nutr* 52: 75-84
- Grama CN Suryanarayana P, Patil MA, Raghu G, Balakrishna N, Ravi Kumar MNV, Reddy GB**.** (2013) Efficacy of biodegradable curcumin nanoparticles in delaying cataract in diabetic rat model. *PLoS One*
- Vasireddy V, Jamison, U. B. Kompella, R. Kadam, V. Joseph, J. H. Lin, G. B. Reddy and R. Ayyagari (2011) Rescue of photoreceptor degeneration by curcumin in transgenic rats with P23H rhodopsin mutation. *PLoS One* 6: e21193.
- Kavitha K, Thiyagarajan P, Rathna J, Nagini S. Chemopreventive effects of diverse dietary phytochemicals against DMBA-induced hamster buccal pouch carcinogenesis via the induction of Nrf2-mediated cytoprotective antioxidant and phase II detoxifying enzymes. *Biochimie* 95:1629-1639.
- Vidya Priyadarsini R, Neeraj Kumar, Imran Khan, Thiyagarajan, P, Kondaiah, P, Nagini S. (2012) Gene expression signature of DMBA-induced hamster buccal carcinomas: Modulation by chlorophyllin and ellagic acid. *PLoS One* 7: e34628.
- Thiyagarajan P, Senthil Murugan R, Kavitha K, Anitha P, Prathiba D, Nagini S. (2012) Dietary chlorophyllin inhibits the canonical NF-κB signaling pathway and induces intrinsic apoptosis in a hamster model of oral oncogenesis. *Food Chem Toxicol.* 50:867-876.
- Vidya Priyadarsini R, Nagini S. (2012) Cancer chemoprevention by dietary phytochemicals: promises and pitfalls. *Curr Pharm Biotechnol* 13:125-136.
- Nagini S, Vidya Priyadarsini R, Veeravarmal V, Mishra RK. (2012) Chlorophyllin abrogates canonical Wnt/□-catenin signaling pathway and angiogenesis to inhibit the development of DMBA-induced hamster cheek pouch carcinomas. *Cell Oncol.* 35: 385-395.
- Akileshwari C, Muthenna P, Nastasijević B, Joksić G, Petrash JM, Reddy GB. (2012) Inhibition of aldose reductase by *Gentiana lutea* extracts. *Exp Diabetes Res*. 147965.
- Muthenna P, Akileshwari C, GB. Reddy (2012) Ellagic acid, a new antiglycating agent: its inhibition of Nε-(carboxymethyl) lysine. *Biochem J.* 442:221-230.
- Muthenna P, Akileshwari C, Saraswat M, GB Reddy (2012) Inhibition of advanced glycation end-product formation on eye lens protein by rutin. *Br J Nutr.* 107:941-949.
- Reddy GB, Muthenna P, Akileshwari C, Saraswat M, Petrash JM. (2011) Inhibition of aldose reductase and sorbitol accumulation by dietary rutin, *Curr. Sci*. 101, 1191-1197.

Scientific impact of the FUNCFOOD project:

The challenge posed by this project was to enhance the health benefit from diets beyond the traditional nutrients by means of addition of bioactive ingredients. This goal was achieved by the step-wise approach providing robust scientific evidence for selecting active ingredients appropriate for inclusion in functional foods.

By starting with experimental models, the research also included mechanistic aspects, in as much as the impact of protective agents on carcinogen uptake and metabolism in humans was predicted, as well as the mode of action of bioactive ingredients on the prevention of age related diseases.

The results achieved indicated that experimental in vitro and in vivo data could be predictable for humans in the sense that it provides rather qualitative than quantitative detection of preventing activity.

Concerning the results on humans, several of the biomarkers showed evidence for detecting preventive mechanisms of background exposure, e.g. decreasing genotoxic risk, and risk for diabetes complications and CVD.

Assessed by the biomarkers used in the humans in intervention, fresh and freezed-dried blueberry showed convincing evidence for being protective against cancer diabetes complications and CVD.

Based on the overall results of over 300 biomarkers, astaxanthine, tomato extract and chlorophyllin showed probable evidence for being protective against cancer, whereas Gentiana extracts are suggested to have no or limited protective activity to any of the diseases.

The results from the human intervention with all five antioxidants showed protective response at individual level to antioxidant intake only when background levels of chromosomal damage were indicated.

In the cohort 100 individuals of different categories, several life-style factors were identified to influence genotoxic risk. Smoking, alcohol consumption and medical therapy were the most important for enhanced risk for cancer. The most important for protection were antioxidant intake and being a vegetarian.

A tool was constructed for prediction of genotoxic risk which will be further developed and evaluated in forthcoming studies on human cohorts.

Socio-economic impact and social implications

We here provided solid scientific data supporting that selected bioactive compounds in this project can be included in functional foods with potential health benefits for prevention and treatment of age related diseases.

Identification of bioactive compounds for which dubious health claims have been made, can here on scientific grounds be neglected for being used in foods.

We hereby verified that several of the biomarkers used in this project could monitor the beneficial effects of functional food constituents on human health.

Data generated here can be explored in models for risk estimation as a basis for dissemination to the public in order to improve health status.