NEWS & VIEWS

Can Transcriptomics Provide Insight into the Chemopreventive Mechanisms of Complex Mixtures of Phytochemicals in Humans?

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Abstract

Blueberries contain relatively large amounts of different phytochemicals, which are suggested to have chemopreventive properties, but little information is available on the underlying molecular modes of action. This study investigates whole genome gene expression changes in lymphocytes of 143 humans after a 4-week blueberry-apple juice dietary intervention. Differentially expressed genes and genes correlating with the extent of antioxidant protection were identified in four subgroups. The magnitude of the preventive effect after the intervention differed between these four subgroups. Furthermore, subjects in two groups carried genetic polymorphisms that were previously found to influence the chemopreventive response. Pathway analysis of the identified genes showed strong but complex gene expression changes in pathways signaling for apoptosis, immune response, cell adhesion, and lipid metabolism. These pathways indicate increased apoptosis, upgraded growth control, induced immunity, reduced platelet aggregation and activation, blood glucose homeostasis, and regulation of fatty acid metabolism. Based on these observations, we hypothesize that combining transcriptomic data with phenotypic markers of oxidative stress may provide insight into the relevant cellular processes and genetic pathways, which contribute to the antioxidant response of complex mixtures of phytochemicals, such as found in blueberry-apple juice. *Antioxid. Redox Signal.* 20, 2107–2113.

Introduction

ALTHOUGH IT IS WIDELY ACCEPTED that consumption of a diet high in fruit and vegetables provides protection against various chronic diseases, including cancer, diabetes, and cardiovascular diseases, recent prospective epidemiological studies failed to support this (8). Nevertheless, results from animal and *in vitro* studies have provided a wealth of information on potential mechanisms of disease risk reduction by bioactive compounds present in plant-based foods. In this respect, blueberries rank highly among fruits for both quality and quantity of bioactive compounds (4, 6). There is plentiful evidence that blueberries or specific blueberry ingredients exert chemopreventive activities in *in vitro* and in rodent models, but little information is available on the mo-

Innovation

This study is unique in its approach to analyze transcriptomic responses after a human dietary intervention study taking relevant genetic variability in the human population into account. In a large setting, gene expression changes were correlated with markers of oxidative stress to provide insight into the molecular mechanisms of the protective effects of blueberry phytochemicals. This novel approach has successfully demonstrated that the integration of transcriptomic and phenotypic data with the individual genotype may enable the optimization of personalized antioxidant-related preventions that will contribute to food innovation and consumer health.

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lecular pathways underlying their beneficial modes of action, especially in humans.

With the advent of genomic techniques and progress in bioinformatics analysis tools, whole genome approaches can be used to analyze the effect of a specific diet on gene expression to identify and characterize changes in the molecular pathways. By linking conventional markers of effect to whole genome gene expression modulation, so-called phenotypic anchoring, we may improve our understanding of molecular pathways underlying actual changes in biomarkers of disease risk. In addition, analyzing gene expression profiles within subgroups of people, with a specific genetic variant, who showed increased benefit from dietary intake of bioactive compounds, can contribute to this identification of relevant processes.

We applied this approach to blood samples from a large-scale human dietary intervention study ($n=143$), in which the DNA protective properties of blueberry-apple juice were assessed. Furthermore, it was investigated whether subgroups with different genetic polymorphisms relevant for antioxidant responses and biotransformation, differed in their response to the intervention on measured biomarkers of cancer risk (7). It was shown that consumption of one liter of blueberry-apple juice per day for 4 weeks raised the total antioxidant capacity of blood plasma as well as the defense against *ex vivo* induced oxidative DNA damage in lymphocytes detected by the comet assay. A significant reduction in oxidative DNA damage occurred, particularly in individuals carrying a glutathione S-transferase theta-1 (*GSTT1*) or X-ray repair cross-complementing protein 1 (*XRCC1*) wild type. We now apply whole genome transcriptome analyses in lymphocytes collected in this blueberry-apple juice intervention study and hypothesize that combining transcriptomic data with markers of the oxidant defense system will identify genetic pathways modulated by the additional supply of phytochemicals in the diet, which provides more insight into the underlying mechanisms of the antioxidant response in humans.

Affected Cellular Processes After Blueberry-Apple Juice Intervention

We compared gene expression profiles between subgroups responding differently with regard to the observed protection

against *ex vivo* induced oxidative DNA damage after the intervention, as well as between groups of individuals carrying either the wild-type or mutant genotype of the two polymorphisms that were previously shown to affect the antioxidant response to intervention (*i.e.*, *GSTT1* and *XRCC1*). Gene expression data obtained from blood taken from 143 healthy nonsmoking volunteers were compared before and after blueberry-apple juice intake. In addition, associations between transcriptomic responses and differences in oxidative DNA damage were investigated by correlation analyses. The numbers of significantly ($p<0.05$) modulated and correlated genes are presented in Table 1.

The overlap of gene effects found between the four different subgroups is expected to identify the most relevant biological processes that are indicative of the observed preventive action. As shown in Figure 1A and B, the cellular processes, immune response, development, and apoptosis were significantly modulated as well as significantly correlated in all four subgroups (a complete overview of all significantly modulated and correlated pathways and related cellular processes can be found in Supplementary Tables S1 and S2, respectively; Supplementary Data are available online at www.liebertpub.com/ars). Furthermore, overlapping processes, which were significantly modulated and correlated in at least two of the four subgroups, involved cell adhesion, transcription, transport, neurophysiological processes, lipid metabolism, muscle contraction, carbohydrate metabolism, and cystic fibrosis. These processes are of particular relevance as they are strongly regulated by the intervention in terms of statistically significant changes in expression of involved genes and the number of genes modulated after the intervention, but even more as the genes in these pathways showed a linear response in relation to the difference in oxidative DNA damage. (A schematic representation of the data analysis workflow can be found in Supplementary Fig. S1).

In the section below, we further interpreted the biological consequences of the gene expression changes for the most relevant processes: apoptosis, immune response, cell adhesion, and lipid metabolism, as these processes are of particular biological relevance in the context of the pathogenesis of chronic diseases in which oxidative cell damage is involved, for instance, cancer, cardiovascular disease, and diabetes.

TABLE 1. DEFINED SUBGROUPS FOR GENE EXPRESSION ANALYSES

Subgroup	N	Delta mean tail moment (SE) ^a	Significantly modulated subgroup-specific genes ^b	Significantly correlating genes with delta mean tail moment ^c
1) All subjects: study population for which comet data were available	143	-1.6 (0.7)	9087	1660
2) Comet group (TM < -2) subjects with mean different tail moment < -2	71	-7.9 (0.8)	3246	4271
3) GSTT1 wild types	118	-1.9 (0.7)	5610	1245
4) XRCC1 wild types	56	-3.7 (0.9)	1544	1988

^aTail moment: integrative measure for DNA damage in the comet. A negative or positive value indicates a decrease or increase in DNA damage after the intervention, respectively. The value represents the magnitude of the effect.

^bIncludes annotated and nonannotated genes. Significantly differentially expressed gene lists after the intervention were generated using ArrayTrack™ by means of pairwise *t*-tests ($p<0.05$).

^cIncludes annotated and nonannotated genes. Significantly correlating genes ($p<0.05$) were generated using Spearman's correlation analyses in which the gene expression changes for each subject were correlated with the difference in mean tail moment after the intervention as measured by means of the comet assay.

FIG. 1. Venn diagrams created using Venny (5) of GeneGo cellular processes after (A) pairwise *t*-test analyses, and (B) correlation analyses, as found by MetaCore analyses of significantly modulated genes. Analyses included the following: 1. all subjects; 2. comet group tail moment (TM) < -2: subjects demonstrating a significant reduction in oxidative DNA damage upon *ex vivo* challenge of lymphocytes as indicated by difference in mean TM < -2; 3. *GSTT1* wild types: subjects carrying the *GSTT1* wild-type variant; and 4. *XRCC1* wild types: subjects carrying the *XRCC1* wild-type variant. Numbers indicate the amount of cellular processes and characters indicate the type of cellular processes. A: Immune response; B: Development; C: Apoptosis; D: Neurophysiological processes; E: G-protein signaling; F: Transport; G: Muscle contraction; H: Transcription; I: Carbohydrate metabolism; J: Lipid metabolism; K: Translation; L: Cystic fibrosis; M: Cell adhesion; N: Neoplastic processes; O: Proteolysis; P: Cytoskeleton remodeling development; Q: Nucleotide metabolism; R: Cell cycle; S: Amino acid metabolism; T: Reproduction; U: Vitamin and cofactor metabolism; V: Oxidative stress. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Molecular Pathways Responding to the Intervention

Apoptosis

For the modulated apoptosis-related genes, a network was generated to visualize the complex set of connections between the genes (Fig. 2A). Furthermore, the involved bio-

logical processes are added to this network (Fig. 2B) (based on the interpretation of the functions of the modulated target genes gathered from EntrezGene and MetaCore) (a detailed overview of all genes present in this apoptosis network is listed in Supplementary Table S3).

The expression of a number of ligands was increased after the intervention, that is, *IL1B*, *IFN*, and *FASLG*. *FASLG* can bind to both the death receptor TNFRSF1A and FAS, which were both upregulated. In addition, genes encoding for membrane receptors were modulated, such as *TLR4* (upregulated), *IGF1R* (downregulated), and *IFNGR1* (upregulated). Upon binding of ligands to these extracellular receptors, several signaling cascades are initiated, such as PI3K/AKT and G-protein signaling, but also activation of downstream targets by MAP kinases, TRAFs, MTOR, PKC, and calmodulin. Activation of FAS and TNFRSF1A results in the recruitment and activation of the initiator CASP8. Active CASP8 subsequently cleaves and activates CASP7, CASP6, and CASP3. Activation of CASP3 leads to the ultimate death of the cell. Inhibition of the PI3K/AKT signaling cascades results in induction of apoptosis, and a reduction in cell survival and cell cycle progression. Genes involved in this cascade were modulated, for instance, the genes encoding for the G-proteins *GNAS2*, *GNAO1*, *GNG2*, *RACK1*, and *GNB5*, and *PIK3CA*, *PIK3R1*, and PKA (all downregulated). The PI3Ks can also be activated by the FYN, which in turn is activated through the binding of nicotine to CHRNA. The downstream effector protein AKT acts *via* inhibition of FKHR or *via* activation of p70 S6 kinase 1 (RPS6KB1) thereby initiating apoptosis and downregulating cell survival. In addition to the extrinsic death receptor pathway, the intrinsic mitochondrial pathway was modulated. When cytochrome c is released from the mitochondria, it binds to the apoptotic protease activating factor 1 (APAF1), which then binds to procaspase nine forming the apoptosome. This in turn activates the effector CASP3. Associated with mitochondrial regulation of apoptosis are the members of the apoptosis regulator Bcl-2 family. The antiapoptotic genes *BCL2* and *MCL1* genes were downregulated, thereby abolishing their inhibiting effects on apoptosis. Furthermore, *BAK1* and *BAX* were upregulated, which are proapoptotic members of the Bcl-2 gene family involved in initiating apoptosis. Overall, the observed gene expression changes occur in different phases of the apoptotic process, from initiation until execution of the death response, thereby stimulating the induction of apoptosis. This strengthens the evidence for the induction of apoptosis as a cancer prevention mechanism induced by dietary phytochemicals (2).

In the same manner as for the gene expression changes in the apoptotic pathways, we analyzed the following processes: immune response, cell adhesion, and lipid metabolism. In these processes, the full sequence of gene expression modulations occurred at the extracellular membrane level, in the cytoplasm, and in the nucleus, leading to modulation of the expression of the target genes. We will only focus on the interpretation of these gene expression effects at the biological level.

Immune response

A number of genes encoding for cytokines and receptors were found to be differentially expressed; these mediate a

FIG. 2. Shortest path network of genes involved in apoptosis related pathways developed in MetaCore including the affected biological processes. (A) Network description: Shortest path network prefiltered on a subcellular level indicating the localization of gene expression of a selection of genes modulated in apoptosis-related pathways. Those genes for which the gene product eventually will result in a cellular effect were defined as target genes and grouped at the base of the network. The color of the upper right small circle at each network object indicates whether the gene was upregulated (red) or downregulated (blue). Detailed information on the symbols can be found at www.genego.com/pdf/MC_legend.pdf. Gene Symbols, full names and information on the function of the genes, including their full name can be found at EntrezGene (www.ncbi.nlm.nih.gov/gene/). The expression of a number of ligands was increased, that is, *IL1B*, *IFN*, and *FASLG*. *FASLG* can bind to both the death receptor *TNFRSF1A* and *FAS*, which were both upregulated. In addition, more cell type-specific genes encoding for membrane receptors were modulated, such as *TLR4* (upregulated), beta-2 adrenergic receptor (upregulated), *IGF1R* (downregulated), and *IFNGR1* (upregulated). Upon binding of ligands to these extracellular receptors, several signaling cascades are initiated, such as PI3K/AKT and G-protein signaling, but also activation of downstream targets by MAP kinases, TRAFs, MTOR, PKC, and calmodulin. Activation of FAS and TNFRSF1A results in the recruitment and activation of the initiator CASP8. Active CASP8 subsequently cleaves and activates CASP7, CASP6, and CASP3. Activation of CASP3 leads to the ultimate death of the cell. Inhibition of the PI3K/AKT signaling cascades results in induction of apoptosis, cell survival, and cell cycle progression. Genes involved in this cascade were modulated, that is, *GNAS2*, *GNAO1*, *GNG2*, *RACK1*, and *GNB5* (all encoding G-proteins), and *PIK3CA*, *PIK3R1*, and *PKA* (all downregulated). The PI3Ks can also be activated by FYN, which in turn is activated through binding of nicotine to the CHRNA. The downstream effector protein AKT acts *via* inhibition of FKHR or *via* activation of p70 S6 kinase 1 (RPS6KB1), thereby initiating apoptosis and downregulating cell survival. In addition to the extrinsic death receptor pathway, the intrinsic mitochondrial pathway was modulated. Associated with mitochondrial regulation of apoptosis are the members of the apoptosis regulator Bcl-2 family. The antiapoptotic genes *BCL2* and *MCL1* were downregulated, thereby abolishing their inhibiting effects on apoptosis. Furthermore, *BAK1* and *BAX* were upregulated, which are proapoptotic members of the Bcl-2 gene family involved in initiating apoptosis. **(B)** Affected biological processes based on the interpretation of gene annotation and function retrieved from EntrezGene (www.ncbi.nlm.nih.gov/gene/) and MetaCore. The color of the square indicates whether the process will be activated (red) or deactivated (green). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

number of similar downstream signaling pathways that are critical for developmental regulation, growth control, and homeostasis. These include the JAK/STAT pathway, the PI3K/AKT pathway, and NF- κ B pathway. Target genes of JAK/STAT include *BCL2* and *MCL1*, which were downregulated, possibly leading to the induction of apoptosis. In addition, *PKR*, *MXA*, *OAS1*, -2, and -3 were upregulated, thereby initiating antiviral and antitumor activities from the innate and adaptive immune system. PI3K/AKT signaling involved downregulation of target genes *FKHR*, *p27KIP1*, *14-3-3*, and *BCL2*, and upregulation of *GRB2* and *PKC*. Overall, these changes indicate a stimulation of cell growth. *IL8* was inhibited, which is a major target gene of the NF- κ B pathway, leading to reduced angiogenesis, cell proliferation, and survival, and to reduced tumorigenesis and metastasis. Furthermore, *HIF1A* and beta-catenin, which are target genes of IL8, were also downregulated, potentially resulting in reduced cell growth and adhesion. Overall, we show a strong and broad effect of immune response-related genes involved in several important signaling pathways, in favor of disease prevention.

Cell adhesion

Several cell surface receptors were modulated after the intervention; these transmit signals from outside the cell into the cell *via* several signaling cascades, among which are G-protein signaling, PIK3 signaling, cAMP protein kinase signaling, and tyrosine kinase signaling. Target genes that were affected include *RHOA*, *RAC1*, *VAMP3*, *RAP-1B* (downregulation), and *CBFB/MYH11 fusion protein* (upregulation); these play a role in several processes involved in platelet aggregation, that is, formation of lamellipodia, platelet shape change, granule secretion, and positive regulation of alpha-II/beta-3 integrins. The gene expression changes may lead to an inhibition of these processes and subsequently to a reduction in platelet aggregation, which is correlated with a reduced risk of thrombus formation in the blood and subsequent risk of infarction.

Lipid metabolism

Insulin plays a major role in the affected pathways by regulating glucose and fatty acid metabolism. In the present study, the gene encoding for insulin was upregulated. In addition, members of the related intracellular signaling pathway PI3K/AKT were affected, leading to modulation of the genes encoding for the transcription factors PAX3/FKHR fusion, SREBP1 nuclear, and SP1. Ultimate target genes that were modulated include *PPCKM*, *PDH beta*, and *G6PC*, which are involved in glucose metabolism; and *CISY*, *Acyl-CoA synthetase*, and *FADS2*, which are involved in fatty acid metabolism. Upregulation of *PPCKM* and *G6PT* could result in initiation of glycolysis and gluconeogenesis and pyruvate metabolisms, resulting in homeostasis of blood glucose levels. This is important in the reduction of the risk of diabetes mellitus. Induction of *CISY* and *Acyl-CoA synthetase*, and *FADS2* may lead to an increase in lipid biosynthesis and fatty acid degradation, and unsaturation of fatty acids, respectively, thereby regulating lipogenesis. These changes could contribute to an adequate fatty acid metabolism, which is of importance in reducing the risk of obesity and type 2 diabetes mellitus.

Concluding Remarks

We identified the relevant mechanistic pathways involved in the chemopreventive action of blueberry phytochemicals by analyzing gene expression profiles after a dietary intervention. We identified subgroups of individuals that either showed differences in their antioxidant responses to the intervention or had different polymorphisms in *GSTT1* or *XRCC1*. Polymorphisms in these two genes were found to be strong determinants of the chemopreventive response. We identified previously unreported genes, which influence the role of specific signaling pathways, such as JAK/STAT, PI3K/AKT, and NF- κ B, in cellular processes that are linked to chronic diseases such as cardiovascular disease, diabetes, and cancer. These results show that the application of transcriptome analyses in a human dietary intervention study is a promising approach to identify mechanistic pathways, which are linked to the prevention of oxidative stress, thereby contributing to our understanding of phytochemical-induced human health effects.

Notes

Materials and Methods

Subjects and study design

The present study on gene expression changes is a follow-up of previously performed work on the evaluation of the antioxidative and antimutagenic effects of a 4-week blueberry-apple juice human dietary intervention study (7). In short, 168 healthy nonsmoking volunteers (114 females and 54 males; aged 18–45 years), were recruited through advertisements in local newspapers. Inclusion of the subjects was based on questionnaires, considering health status, dietary habits, including the use of supplements, profession, use of medication, and other lifestyle factors. The study started with a 5-day flavonoid washout period, which was followed by a 4-week intervention period, during which, subjects consumed one liter of custom-made blueberry-apple juice mixture per day. Characterization of the juice has been carried out by Plant Research International (Wageningen UR, The Netherlands). Detailed phytochemical content is listed in Table 2. Before and after the intervention period, blood was

TABLE 2. PHYTOCHEMICAL CHARACTERIZATION OF BLUEBERRY/APPLE JUICE

	Blueberry apple juice (μ g/ml)
Caffeic acid derivative	74.8
Chlorogenic acid	1235
Unknown anthocyanin	20
Delphinidine-3-glucoside	58.6
Delphinidine-3-rutinoside	27.5
Cyanidine-3-glucoside	38.8
Peonidine-3,5-diglucoside	58.3
Pelargonidine-3-glucoside	18.6
Peonidine-3-glucoside	109
Malvidine-3-glucoside	74.3
Quercetine-3-glucoside	450
Quercetine	81.4

collected from which, plasma and lymphocytes were isolated; these were used for determination of several phenotypic and genotypic parameters. Lymphocytes were challenged *ex vivo* with hydrogen peroxide and analyzed for oxidative DNA damage using the alkaline comet assay. From 143 subjects (98 females and 45 males; aged 18–45 years), comet data are available. All participants were genotyped for 34 single-nucleotide polymorphisms associated with oxidative stress, biotransformation, and DNA repair. This study was approved by the medical ethics committee of Maastricht University and the Academic Hospital Maastricht. Written informed consent was obtained from the participants before the start of the study.

Whole Genome Gene Expression Profiling Using Microarrays

RNA isolation

Lymphocytes isolated from the blood drawn from each participant before and after the intervention, were lysed with the TRIzol[®] reagent (Invitrogen, Breda, The Netherlands) and stored at -80°C until RNA extraction. Samples were thawed at room temperature, and total RNA was extracted according to the manufacturer's instructions. The RNeasy[®] Mini Kit was used to purify total RNA from salts and residual DNA (Qiagen, Venlo, The Netherlands). The quantity and purity of RNA were measured spectrophotometrically. RNA integrity was assessed by automated gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples had an OD 260/280 ratio between 1.8 and 2.0 and held an RNA integrity number >8 .

Target preparations, hybridizations, and scanning procedures

Cyanine 3 (Cy3)- and Cyanine 5 (Cy5)-labeled cRNA probes were generated using the Two-Color Microarray-Based Gene Expression Analysis kit from Agilent Technologies (Amstelveen, The Netherlands) according to the manufacturer's protocol. To rule out any possible dye effect, samples from each subject before and after the intervention were randomly labeled with Cy3 or Cy5 (PerkinElmer, Norwalk, CT). Hybridization was carried out on Agilent 4x44K Whole Human Genome Oligo microarrays. In the paired study design, each subject acted as its own control, meaning that the fluorescent-labeled cRNAs of each subject before and after the intervention were mixed and hybridized on one chip. Hybridization, washing, and scanning were performed as described by Hebels *et al.* (3).

Image analyses, data preparation, and normalization

The images obtained (resolution $5\text{ }\mu\text{m}$, 16 bit tiff image) were processed with GenePix[®] Pro 6.0 software (Molecular Devices, Sunnyvale, CA) to measure mean signal intensities for spots and local backgrounds. Poor spots were flagged automatically and covered $<5\%$ of total spots. Quality control was performed in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria). Data were imported into ArrayTrack[™] version 3.4.5 (U.S. Food and Drug Administration, Jefferson, AR) for further processing. Background-corrected mean in-

tensities were log base 2 transformed and LOWESS normalization was applied.

The gene expression data of the present study have been deposited in NCBI's Gene Expression Omnibus (GEO) and are available through GEO series GSE41781 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hrsjoimgssuete&acc=GSE41781). The GEO is a public repository that archives and freely distributes microarray, next-generation sequencing, and other forms of high-throughput functional genomic data submitted by the scientific community. In addition to data storage, a collection of web-based interfaces and applications are available to help users query and download the studies and gene expression patterns stored in GEO (1). For more information about various aspects of GEO and how to access data, please see the documentation listings and publications on the GEO website (www.ncbi.nlm.nih.gov/geo/info/).

Microarray data analyses

Subgroups were created according to the magnitude of the effect in the comet assay. In addition, lymphocytes obtained from *GSTT1* or *XRCC1* wild-type variants showed an intervention-related decrease in single-strand breaks as measured by the comet assay. In total, four subgroups were defined for which extensive data analyses were performed (i) all subjects: study population for which comet data were available. This group showed an overall reduction in mean tail moment (TM) (mean $\pm$ SE: -1.6 ± 0.7); (ii) comet group TM < -2 : strong responders in the comet assay. This group showed a reduction in mean TM < -2 , (mean $\pm$ SE: -7.9 ± 0.8); (iii) *GSTT1* wild-type variants; and, (iv) *XRCC1* wild-type variants. Looking at the overlap of transcriptomic responses between the four differently defined subgroups enables the identification of the relevant pathways and biological processes, which are indicative for the chemopreventive action of a fruit juice rich in phytochemicals, as these four subgroups show an intervention-related protective effect against the induction of subsequent oxidative DNA damage.

Using ArrayTrack, for each group, gene lists comprising genes that were significantly differentially expressed after the intervention were generated by means of pairwise *t*-tests ($p < 0.05$) in which, each subject acted as its own control (Table 1). The false discovery rate at this *p*-value was $<15\%$.

To correlate gene expression data for the different subgroups with the measured difference in mean TM as the parameter of DNA damage, Spearman's rank correlation analyses were performed. Only genes present in at least 70% of the subjects were used. Before correlation analysis, missing values were imputed by finding the *k* nearest neighbors (*k* was set to 15), using a Euclidean metric, and imputing the missing elements by averaging the (nonmissing) elements of its neighbors. The online Gene Expression Profile Analysis Suite (GEPAS, <http://gepas.bioinfo.cipf.es/>) was used to perform the Spearman's correlation analyses (Table 1).

To identify the involvement of the genes in specific cellular GeneGo pathways, the gene sets were transferred into the online commercially software suite MetaCore[™] version 6.1 (GeneGo, San Diego, CA). Pathways with a *p*-value < 0.05 were considered to be significantly modulated.

Gene networks based on a select number of genes for a selection of cellular processes were created in MetaCore using the Dijkstra's shortest path algorithm. The resulting networks were prefiltered on a subcellular level indicating the localization of gene expression. These subcellular levels comprise the extracellular membrane, cytoplasm, and nucleus. For each network, those genes for which the gene product eventually will result in a cellular effect were defined as target genes and grouped at the base of the network. Based on the interpretation of gene annotation and function retrieved from EntrezGene (www.ncbi.nlm.nih.gov/gene/) and MetaCore, the modulated target genes were linked to biological processes. Full names of the abbreviated genes, including a description of their function, can be gathered from EntrezGene (www.ncbi.nlm.nih.gov/gene/).

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References

1. Edgar R, Domrachev M, and Lasha AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207–210, 2002.
2. Gossiau A and Chen KY. Nutraceuticals, apoptosis, and disease prevention. *Nutrition* 20: 95–102, 2004.
3. Hebel DG, Jennen DG, Kleinjans JC and de Kok TM. Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis. *Toxicol Sci* 108: 290–300, 2009.
4. Moyer RA, Hummer KE, Finn CE, Frei B, and Wrolstad RE. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: vaccinium, rubus, and ribes. *J Agric Food Chem* 50: 519–525, 2002.
5. Oliveros JC. VENNY. An interactive tool for comparing lists with Venn Diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>. (Last accessed July 1, 2013).
6. Vinson JA, Su X, Zubik L, and Bose P. Phenol antioxidant quantity and quality in foods: fruits. *J Agric Food Chem* 49: 5315–5321, 2001.
7. Wilms LC, Boots AW, de Boer VC, Maas LM, Pachen DM, Gottschalk RW, Ketelslegers HB, Godschalk RW, Haenen GR, van Schooten FJ, and Kleinjans JC. Impact of multiple genetic polymorphisms on effects of a 4-week blueberry juice intervention on *ex vivo* induced lymphocytic DNA damage in human volunteers. *Carcinogenesis* 28: 1800–1806, 2007.
8. World Cancer Research Fund/American Institute for Cancer Research. *Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective*. Washington DC: AICR, 2007, p. 517.

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Abbreviations Used

APAF1 = apoptotic protease activating factor 1
Cy3 = cyanine 3
Cy5 = cyanine 5
GEO = Gene Expression Omnibus
GSTT1 = glutathione S-transferase theta-1
TM = tail moment
XRCC1 = X-ray repair cross-complementing protein 1