# Whole-genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells

**Mutagenesis** vol. 26 no. 6 pp. 753–761, 2011
Advance Access Publication 1 July 2011
doi:10.1093/mutage/ger043

# Whole-genome gene expression modifications associated with nitrosamine exposure and micronucleus frequency in human blood cells

**Dennie G. A. J. Hebels, Danyel G. J. Jennen, Marcel H. M. van Herwijnen, Edwin J. C. Moonen, Marie Pedersen[1], Lisbeth E. Knudsen[1], Jos C. S. Kleinjans and Theo M. C. M. de Kok**

Department of Toxicogenomics, Maastricht University, Universiteitssingel 50, PO Box 616, 6200 MD Maastricht, The Netherlands and [1]Department of Public Health, University of Copenhagen, Øster Farimagsgade 5, PO Box 2099, DK-1014 Copenhagen K, Denmark.

*To whom correspondence should be addressed. Department of Toxicogenomics, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands. Tel: +31 43 3881088; Fax: +31 43 3884146; Email: d.hebels@maastrichtuniversity.nl

*Received on April 8, 2011; revised on May 25, 2011; accepted on May 31, 2011*

***N*-nitroso compounds (NOCs) are suspected human carcinogens and relevant in human exposure. NOCs also induce micronuclei (MN) formation *in vivo*. Since lymphocytic MN represent a validated biomarker of human cancer risk, establishing a link between NOC exposure and MN frequency in humans and concurrently investigating associated transcriptomic responses may provide crucial information on underlying molecular mechanisms that predispose to carcinogenicity. We used lymphocytes, from adult females participating in the pan-European biomarker research project NewGeneris, as a surrogate tissue for analysing such potentially carcinogenic gene expression and MN formation events in target organs. To assess NOC exposure, urine samples were analysed for marker nitrosamines. NOC excretion levels and MN frequency were subsequently linked to peripheral blood transcriptomics. We demonstrated a significant association between MN frequency and urinary NOCs ($r = 0.41$, $P = 0.025$) and identified modifications in among others cytoskeleton remodeling, cell cycle, apoptosis and survival, signal transduction, immune response, G-protein signaling and development pathways, which indicate a response to NOC-induced genotoxicity. Moreover, we established a network of genes, the most important ones of which include FBXW7, BUB3, Caspase 2, Caspase 8, SMAD3, Huntingtin and MGMT, which are involved in processes relevant in carcinogenesis. The modified genetic processes and genes found in this study may be of interest for future investigations into the potential carcinogenic risk associated with NOC exposure in humans.**

## Introduction

Research on human exposure to *N*-nitroso compounds (NOCs) has received much attention since it was shown in the 1960's that many NOCs are carcinogenic in rats (1). Most NOCs have been classified as probable or possible human carcinogens by the International Agency for Research on Cancer (2), and many have been identified in foodstuffs, such as cured meat and beer, particularly NOCs belonging to the nitrosamine class. Policy measures to decrease the level of NOCs in these products have led to a significantly lower dietary exposure (3). NOCs may also be formed endogenously in the stomach and colon out of the reaction between nitrate and amines or amides; this may actually result in higher colonic exposure than caused by food-borne NOC and may thus represent an important carcinogenic risk (4,5). It remains difficult, however, to assess the actual human cancer risk as human exposure is relatively low and information on the possible (pre-) carcinogenic effects of NOCs in humans is sparse. Although there have been numerous reports on genotoxic and mutagenic properties of NOCs *in vitro* in, for example, the sister chromatid exchange assay and Ames test (6), this does not necessarily imply a carcinogenic risk for intact humans, especially, since it is difficult to determine the relationship between NOC-induced genotoxicity or mutagenicity and the associated carcinogenicity of these compounds (7). In several epidemiological studies, human NOC exposure has been associated with increased cancer risks of the stomach, esophagus, bladder and colon (8,9), in particular in association with dietary intake of food items with relatively high levels of NOC precursors or nitrosating agents (4,10,11). There is, however, still no consensus on whether NOCs actually are human carcinogens.

In an attempt to assess the potential carcinogenic effect of NOCs in humans, with particular interest for NOC-induced molecular mechanisms in the colon, we previously investigated gene expression changes in the human colon adenocarcinoma cell line Caco-2 (12). Indeed, we identified a large number of NOC-modified molecular pathways involved in processes that may contribute to the carcinogenic potential of NOCs in humans, including pathways crucial in differentiation and proliferation. Therefore, in the present study, we have evaluated whether NOC exposure in human subjects induces gene expression responses, which provide insights in their possible human carcinogenicity. We have previously used genomic analyses to improve our understanding of childhood cancer risk associated with exposure to air pollution (13) and have also investigated differences in gene expression levels in adults subject to environmental burdens where associations were found of gene expression with blood and urinary measures of environmental carcinogens (14). In this study, we used a similar approach by analysing the excretion of two marker nitrosamines, i.e. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopiperidine (NPIP) (15), in urinary samples from pregnant mothers participating in the Danish pilot study of the multidisciplinary research project NewGeneris (www.newgeneris.org) (16,17). Both nitrosamines are known to be carcinogenic in test animals, may occur in several foodstuffs and can be formed endogenously (18–20). Moreover, since NOCs cause micronuclei (MN) formation *in vivo* and MN represent a validated predictive biomarker of carcinogenic risk (21–23), MN analysis in

peripheral lymphocytes provides an interesting marker to associate with human NOC exposure. NOC excretion levels and MN frequency were subsequently linked to peripheral blood transcriptomics to gain mechanistic insight into the relationship between human NOC exposure and MN formation in relation to human carcinogenesis.

## Materials and methods

*Study population*

Potential study participants were identified from the pregnancy medical records from the University Hospital of Copenhagen and the inclusion criteria were (i) no chronic health complications, e.g. diabetes; (ii) >18 years; (iii) no private commercial banking of cord blood; (iv) signed written informed consent and (v) capacity to understand the informed consent. Eligible participants were (i) non-smoking pregnant women, (ii) with no residential environmental tobacco smoke exposure and (iii) who spent the majority of the time at home. The Ethics Committee of the Capital Region of Denmark (Reference No. J. Nr. H-KF-01-327603) and the Danish Data Protection Agency (Reference No. J. Nr. 2007-41-0415) reviewed and approved the studies prior to initiation. The study population consisted of a subset of 32 pregnant women from a cross-sectional biomonitoring study described previously (17), who were very homogenous in age (32.4 ± 3.7 years) and food intake. Further details on the characteristics of the study population can also be found there.

*Biological sample collection*

Blood samples (~50 ml) were drawn in heparinised tubes (Becton Dickinson, Oxford, UK) by venipuncture in the morning hours, between 7 and 11 a.m. and 1–2 h before their planned caesarean section. The women had been fasting for ~6 h. To stabilise RNA, 0.4 ml of whole blood was mixed with 1.2 ml RNAlater (Ambion, Naerum, Denmark) and stored at −20°C for the first 24 h followed by a longer term storage at −80°C for 3 years until use. Twenty-four hour urine was collected in 2-l containers 1–5 days before child delivery and ~10 g of NaOH was added to the urine to avoid degradation of NOC. Aliquots of 50 ml were first stored at −20°C (for ~2 years) and subsequently at −80°C (~6 months) until analysis.

*Gas chromatography–mass spectrometry analysis of NDMA and NPIP in urine*

NDMA and NPIP were determined by gas chromatography–mass spectrometry (GC–MS) as previously described (24). In short, urine samples from donors were thawed and extracted with dichloromethane (750 µl/14 ml urine). Then 1 µl of the extracted dichloromethane solution was injected into the GC–MS system. Molecular ions were detected using a resolution of 3000 and quantification was performed by a calibration curve for each of the two nitrosamines, using standards dissolved in urine and extracted similarly to adjust for a possible loss of nitrosamines during workup. Recovery of NOC was 40% (NDMA) and 90% (NPIP) (detection limit: 0.05 ng/ml urine). Total urinary NOC levels (NDMA + NPIP) were expressed in nanomoles per millimole creatinine. Creatinine was measured by the Jaffe's kinetic alkaline picrate method. Creatinine and picric acid were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Creatinine concentrations were determined spectrophotometrically at a wavelength of 500 nm.

*Cytokinesis-block micronucleus assay*

MN frequencies per 1000 once-divided binucleated cells were analysed using the cytokinesis-block micronucleus (CBMN) assay (25,26). Approximately 6 h after collection from donors, whole blood cultures were initiated in duplicate. At 72 h following phytohaemagglutinin stimulation, cells were harvested, fixed, applied to slides and Giemsa stained as described before (17) and subsequently scored (~2000 binucleated cells per donor) using a light microscope at ×400 magnification following the criteria for scoring MN set in the international collaborative project on MN frequency in human populations (HUMN) (27). For two subjects, no MN could be scored.

*Microarray hybridisation and quality control*

RNA was isolated from RNAlater-stabilised whole blood according to the manufacturer's protocol. Microarray hybridisation was performed as described previously (12). In short, dye-labeled cRNA (Cy3) was synthesised following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies, Amstelveen, The Netherlands). Samples were hybridised on Agilent 4x44K Whole Human Genome microarrays. After scanning the microarray slides, using settings described before (12), bad and empty spots were flagged in GenePix Pro (version 6.0; Molecular Devices, Sunnyvale, CA, USA). Quality control was performed in R (version 2.10.1; The R Foundation for Statistical Computing, Vienna, Austria). Quantile normalisation and data processing were performed in ArrayTrack (version 3.4; NCTR, Jefferson, AR). Log2-transformed spot intensities or ratios were used for further analyses. For two subjects, microarrays did not pass quality control.

*Statistics and microarray data analysis*

Associations between urinary NOC excretion levels and MN frequencies were investigated using Pearson's correlation analysis after log2 transformation of urinary excretion values to obtain a normally distributed data set. Normality of the individual parameters was checked with the Kolmogorov–Smirnov test. Only one outlier in the correlation analysis was identified, having an absolute studentised residual >2 and a Cook's distance >4/*n* and therefore excluded (28). A $P < 0.05$ was considered significant.

ArrayTrack was used to find significantly modulated genes (two-tailed Welch *t*-test, $P < 0.05$) between groups of subjects with the highest and lowest total urinary NOC excretion or MN frequency. For this comparison, the highest and lowest tertiles of total urinary NOC excretion ($n = 10$ per tertile) or MN frequency ($n = 9$ per tertile) were selected since they displayed the most significant difference (in contrast to quantiles, etc.). Differentially expressed genes between the highest and lowest tertiles of either total urinary NOC excretion or MN frequency were identified by combining a log ratio ranking (absolute log2 ratio >0.5) with a non-stringent *P* cut-off of 5%, as suggested by Shi *et al.* (29). Furthermore, only genes detected on the microarray and/or not flagged as a bad spot in >70% of subjects were used in this analysis. Genes were subsequently imported into MetaCore™ (GeneGo, San Diego, CA, USA) to identify the involvement of differentially expressed genes in specific cellular pathways. Pathways with a $P < 0.05$ were considered significantly modulated.

Gene expression data (log2-transformed spot intensities) were also used to perform Spearman's rank correlation analyses with levels of urinary NOC excretion and MN frequency using the online Gene Expression Profile Analysis Suite (GEPAS, http://gepas.bioinfo.cipf.es/). Only genes that were detected on the microarray and/or not flagged as a bad spot in >70% of subjects were used in the correlation analysis. Prior to correlation analysis, missing values were imputed in GenePattern (http://www.broad.mit.edu/cancer/software/genepattern/, version 3.1) by finding the *k* nearest neighbors ($k = 15$, Euclidean metric) and imputing missing elements by averaging non-missing elements of its neighbors. Significantly correlating genes ($P < 0.05$) were further analysed in MetaCore for pathway involvement. A gene network based on a select number of genes was created in MetaCore using Dijkstra's shortest paths algorithm (30).

The gene expression data discussed in this publication have been deposited in NCBI's (National Center for Biotechnology Information) Gene Expression Omnibus (GEO) and are available through GEO Series accession number GSE23919 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23919).

## Results

### *Correlation analysis*

To link NOC exposure with carcinogenic risk, we determined whether there was a relationship between total urinary NOC excretion (i.e. NDMA + NPIP), as a marker of exposure, and lymphocytic MN frequency as a biomarker of effect. We found a statistically significant positive correlation between MN and total urinary NOC excretion ($r = 0.41$, $P = 0.025$, Figure 1).

### *NOC exposure-related gene expression*

We subsequently investigated transcriptomic responses in lymphocytes to identify gene expression modulations that can be related to NOC exposure, which may enable identifying underlying molecular modes of action. Since women in the outer tertiles of total urinary NOC excretion ($n = 10$ per tertile) displayed the most significant difference in excretion levels ($P < 0.01$, results not shown), differentially expressed genes in whole blood between the highest and lowest tertile of total urinary NOC excretion were identified. Pathway analysis with the differentially expressed genes revealed a list of significantly modulated pathways ($P < 0.05$) between the outer tertile groups (Table I). Differences in levels of NOC excretion are mainly associated with modifications in amino acid metabolism, apoptosis and survival, cell adhesion and a few other signaling and metabolism pathways.

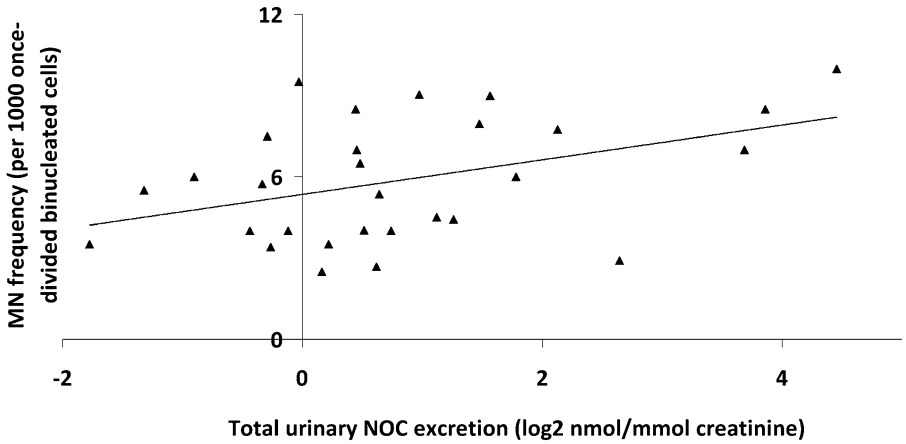


**Fig. 1.** Pearson's correlation plot of log2-transformed total urinary NOC excretion versus MN frequency (per 1000 once-divided binuclear cells) in lymphocytes ($r = 0.41$, $P = 0.025$, $n = 29$).

**Table I.** GeneGO pathways significantly modulated by genes differentially regulated between subjects based on highest and lowest tertile of total urinary NOC excretion

| Process | Pathways involved | % genes[a] | *P*-value |
|---|---|---|---|
| Amino acid metabolism | Selenoamino acid metabolism | 22 | 0.021 |
| | Glycine, serine, cysteine and threonine metabolism | 12 | 0.026 |
| | Tyrosine metabolism p.2 (melanin) | 15 | 0.043 |
| Apoptosis and survival | Endoplasmic reticulum stress response pathway | 13 | 0.003 |
| | Regulation of apoptosis by mitochondrial proteins | 11 | 0.035 |
| | Caspase cascade | 11 | 0.035 |
| | Cytoplasmic/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim | 10 | 0.045 |
| Blood coagulation | Blood coagulation | 14 | 0.016 |
| Cell adhesion | ECM remodeling | 14 | 0.006 |
| | Plasmin signaling | 14 | 0.016 |
| Development | Thrombopoetin signaling via JAK-STAT pathway | 16 | 0.012 |
| Immune response | IL-2 activation and signaling pathway | 10 | 0.045 |
| Neurophysiological process | GABAergic neurotransmission | 20 | 0.026 |
| | Gamma-aminobutyrate (GABA) biosynthesis and metabolism | 15 | 0.043 |
| Regulation of lipid metabolism | RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR | 15 | 0.043 |
| Sulfur metabolism | Sulfur metabolism | 14 | 0.050 |

Group sizes for tertiles were $n = 10$.
[a]Percentage of significantly correlated genes compared to the total number of genes in the pathway ($P < 0.05$).

To explore gene expression differences associated with NOC exposure in more detail, we also analysed Spearman's rank correlations between individual gene expression data and total urinary NOC values. Significantly correlating genes ($P < 0.05$) were analysed for their involvement in biological pathways using MetaCore (Table II). NOC excretion was again associated with amino acid metabolism and apoptosis and survival. One pathway involved in cell cycle regulation was also identified. Most of the other modified pathways were involved in cytoskeleton remodeling, development and G-protein signaling.

*MN frequency-related gene expression*

Similar to the analyses performed with urinary NOCs, we associated differentially expressed genes with MN frequencies in the highest versus lowest MN tertile ($n = 9$ per tertile) and performed a correlation analysis of MN levels and gene expression data. As can be expected from the significant correlation between urinary NOCs and MN frequency, there was a strong overlap between subjects in the outer tertiles (i.e. the highest and lowest tertile combined) of the NOC excretion group (total outer tertile $n = 20$) and MN frequency group (total outer tertile $n = 18$) of 13. Significantly modulated genes between the highest and lowest tertiles or significantly correlating genes ($P < 0.05$) were subsequently analysed in MetaCore. In Tables III and IV, respectively, the results of the tertile and correlation analyses are presented. The tertile analysis only identified pathways related to carbohydrate and lipid metabolism. Most of these pathways were also identified in the MN frequency correlation analysis in addition to pathways involved in development and transport and a few other cytoskeleton remodeling, signal transduction, transcription and metabolism-related pathways.

Comparison of Tables I and II with Tables III and IV shows that three pathways are in common for the urinary NOC and MN analyses, which are 'Role of PKA (protein kinase A) in cytoskeleton reorganisation', 'CREB (cAMP (cyclic AMP) response element binding) pathway' and 'RXR (retinoid X receptor)-dependent regulation of lipid metabolism via PPAR (peroxisome proliferator-activated receptor), RAR (retinoic acid receptor) and VDR (vitamin D receptor)'.

**Table II.** GeneGO pathways significantly modulated by genes differentially regulated between subjects correlating with total urinary NOC excretion

| Process | Pathways involved | % genes[a] | *P*-value |
|---|---|---|---|
| Apoptosis and survival | Apoptotic TNF-family pathways | 32 | 0.003 |
| | Caspase cascade | 29 | 0.024 |
| Carbohydrate metabolism | Glycolysis and gluconeogenesis p. 2/Human version | 50 | 0.006 |
| Cell cycle | Regulation of G1/S transition (part 1) | 27 | 0.046 |
| Cytoskeleton remodeling | ACM3 and ACM4 in keratinocyte migration | 44 | 0.022 |
| | Role of PKA in cytoskeleton reorganisation | 33 | 0.023 |
| | ESR1 action on cytoskeleton remodeling and cell migration | 36 | 0.046 |
| Development | Activation of astroglial cells proliferation by ACM3 | 33 | 0.038 |
| | EGFR signaling via PIP3 | 36 | 0.046 |
| G-protein signaling | RhoA regulation pathway | 44 | 0.000 |
| | Regulation of CDC42 activity | 30 | 0.019 |
| | Regulation of RAC1 activity | 28 | 0.030 |
| | Rac2 regulation pathway | 30 | 0.039 |
| Immune response | Antigen presentation by MHC class II | 57 | 0.008 |
| Neurophysiological process | Long-term depression in cerebellum | 42 | 0.014 |
| | Netrin-1 in regulation of axon guidance | 40 | 0.033 |
| | Glutamate regulation of dopamine D1A receptor signaling | 36 | 0.046 |
| Sulfur metabolism | Sulfur metabolism | 31 | 0.049 |
| Transcription | CREB pathway | 33 | 0.038 |
| Transport | Alpha-2 adrenergic receptor regulation of ion channels | 33 | 0.038 |

Group size for correlation analysis was $n = 30$.
[a]Percentage of significantly correlated genes compared to the total number of genes in the pathway ($P < 0.05$).

**Table III.** GeneGO pathways significantly modulated by genes differentially regulated between subjects based on highest and lowest tertile of MN frequency

| Process | Pathways involved | % genes[a] | *P*-value |
|---|---|---|---|
| Carbohydrate metabolism | Fructose metabolism | 17 | 0.027 |
| | Glycolysis and gluconeogenesis | 15 | 0.040 |
| Regulation of lipid metabolism | Phospholipid metabolism | 38 | 0.006 |
| | RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR | 21 | 0.031 |
| | HETE and HPETE biosynthesis and metabolism | 20 | 0.038 |
| | Plasmalogen biosynthesis | 20 | 0.038 |

Group sizes for tertiles were $n = 9$.
[a]Percentage of significantly correlated genes compared to the total number of genes in the pathway ($P < 0.05$).

**Table IV.** GeneGO pathways significantly modulated by genes differentially regulated between subjects correlating with MN frequency

| Process | Pathways involved | % genes[a] | *P*-value |
|---|---|---|---|
| Carbohydrate metabolism | Fructose metabolism | 17 | 0.025 |
| | Glycolysis and gluconeogenesis p. 1 | 20 | 0.036 |
| Cytoskeleton remodeling | Role of PKA in cytoskeleton reorganisation | 22 | 0.011 |
| Development | Role of IL-8 in angiogenesis | 14 | 0.028 |
| | Endothelin-1/EDNRA signaling | 16 | 0.033 |
| | EDG3 signaling pathway | 18 | 0.050 |
| Heme metabolism | Heme metabolism | 19 | 0.018 |
| Regulation of lipid metabolism | Regulation of fatty acid synthase activity in hepatocytes | 38 | 0.006 |
| | Phospholipid metabolism p.1 | 38 | 0.006 |
| | Stimulation of arachidonic acid production by ACM receptors | 14 | 0.028 |
| | RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR | 21 | 0.030 |
| | Inhibitory action of lipoxins and resolvin E1 on neutrophil functions | 20 | 0.036 |
| Signal transduction | PKA signaling | 15 | 0.038 |
| Steroid metabolism | Cortisone biosynthesis and metabolism | 29 | 0.044 |
| Transcription | CREB pathway | 20 | 0.036 |
| Transport | Regulation of degradation of deltaF508 CFTR in CF | 19 | 0.018 |
| | CFTR folding and maturation (norm and CF) | 25 | 0.019 |
| | Regulation of CFTR activity (norm and CF) | 17 | 0.025 |
| | Regulation of degradation of wt-CFTR | 19 | 0.042 |

Group size for correlation analysis was $n = 28$.
[a]Percentage of significantly correlated genes compared to the total number of genes in the pathway ($P < 0.05$).

*MN formation-associated gene network analysis*

We recently presented a network analysis of a list of genes related to MN formation, involved in processes such as spindle assembly and DNA damage control, based on available knowledge from literature (31). These genes were here compared with the NOC excretion and MN frequency-related gene sets and three genes (of a total of 27 genes and three gene complexes) were found to overlap. One of these genes, identified as FBWX7 (F-box and WD repeat domain containing 7), was found in the MN frequency-related gene set and was represented in both the tertile comparison and the correlation analysis. FBXW7 was significantly upregulated in the upper tertile group compared to the lower tertile group (log2 ratio: 0.8) and was positively correlated with MN frequency. The other two genes were found in the NOC excretion-related group and were identified as BUB3 (budding uninhibited by benzimidazoles 3 homolog) and caspase 2. BUB3 was significantly downregulated in the upper tertile group compared to the lower tertile group (log2 ratio: −1.1) and negatively correlated with NOC excretion levels ($P < 0.05$). Caspase 2 was non-significantly upregulated in the upper tertile group compared to the lower tertile group (log2 ratio: 1.8) but significantly and positively correlated with NOC excretion ($P < 0.05$). MetaCore was subsequently used to integrate FBXW7, BUB3 and caspase 2 into a network to visualise their interactions. In Figure 2A, a, network analysis is presented to find the shortest directed paths between genes of interest. The network does not include all known interactions of FBXW7, BUB3 and caspase 2 with other genes since this would generate a too large network. The genes in this network were checked for matches with significantly modulated or correlated genes in the NOC excretion and MN frequency-related gene sets and three matching genes were identified as caspase 8, huntingtin (HTT) and SMAD3 (SMAD family member 3), which were significantly correlated with NOC excretion, thus increasing the potential relevance of the genes in the network in relation to carcinogenicity. Since $O^6$-methylguanine-DNA methyltransferase (MGMT) is an important gene involved in repair of alkyl adducts (18), this gene was screened for significant modification or correlation with NOC excretion and/or MN frequency and found to be significantly upregulated in the MN frequency tertile analysis (log2 ratio: 0.5). Addition of MGMT to the network shows a close interaction with genes already present in the network. In Figure 2B, an overview is presented of possible locations where these seven genes and some of the modified pathways could play a role in the relation between NOC exposure, repair, MN formation and carcinogenesis.

## Discussion

Analysing gene expression modulations associated with known markers of exposure or well-validated phenotypic markers of effect provide an innovative way of assessing environmental health risks in chemically exposed populations of interest. Microarray technology generates a large amount of information, which not only makes it an interesting marker of effect but also provides a complete overview of modified genes and processes that can be helpful in understanding the molecular basis of the effects induced by the compounds under investigation. In the present study, we investigated the possible carcinogenicity of NOCs in humans, using whole blood as a surrogate tissue, by linking transcriptomics with urinary NOC excretion and lymphocytic MN frequency as markers of exposure and effect, respectively.

The significant positive association found between total urinary NOC excretion levels and MN frequencies in lymphocytes indicates a relation between NOC exposure and the induction of genotoxicity (Figure 1). Other studies have also implicated nitrosamines, and NDMA in particular, as a likely contributor to human genotoxicity in surrogate tissues. Formation of $O^6$-methylguanine in human lymphocyte DNA, for example, which is a pre-mutagenic lesion induced by NDMA, was found in a large number of individuals not known to have suffered excessive exposure to methylating agents (32). When there are no evident exogenous sources of NOCs, such as tobacco smoke, most of the genotoxicity induced by NOCs can be attributed to endogenous formation (5), which is most likely the case in this study population since environmental tobacco smoke and ex-smoking did not significantly affect MN levels, as previously published (17). The fact that in our study, MN frequency was associated with exposure to NOCs points towards a possible increased carcinogenic risk associated with NOC exposure given that lymphocytic MN frequency is considered a validated predictor of human carcinogenicity (21). Nevertheless, some reservations should be made with regard to the interpretation of MN scores in relation to *in vivo* exposures as a direct marker of cancer risk (33). These reservations stem mostly from the uncertainty about the origin of the MN formed during the *ex vivo* proliferation of lymphocytes after *in vivo* exposures to environmental genotoxins. In addition, the relationship studied here between MN and NOC exposure or gene expression could be different for MN originating from chromosome breaks and those containing whole chromosomes originating from chromosome malsegregation, which could be distinguished in future studies using centromere probes. It also important to consider the influence of background variables that affect MN formation, such as age and nutritional status, but these were homogenously distributed in this study population as published previously (17) and therefore not likely to have been of great influence. All in all, these results provide further prove that nitrosamines pose a genotoxic risk to humans, which may also contribute to the carcinogenic process.

The relationship between NOC exposure and genotoxicity was further substantiated by establishing the transcriptomic response associated with exposure to NOCs in lymphocytes. Two separate analyses were performed, one focusing on the comparison of subjects between the higher and lower tertile of NOC excretion as a marker of exposure (Table I) and one focusing on genes significantly correlated with NOC excretion across all subjects (Table II). The first analysis allows for the identification of the strongest effects between high- and low-exposure groups while the correlation analysis may capture more subtle effects since all genes, regardless of log2 ratio or significance level, are used. Associations with several apoptosis and survival pathways were identified in both analyses indicating that cell survival is affected by NOCs in humans. A closer examination of the genes involved in these pathways revealed that several pro-apoptotic genes were upregulated in the upper exposure tertile of subjects thus indicating that higher NOC exposure may be related with an increase in apoptosis. This may demonstrate a direct consequence of the genotoxic potency of these compounds. Although *in vitro* exposure of cultured cells requires high millimolar concentrations of NOCs to induce DNA damage or apoptosis (12,34,35), human lymphocytes become apoptotic at micromolar NOC

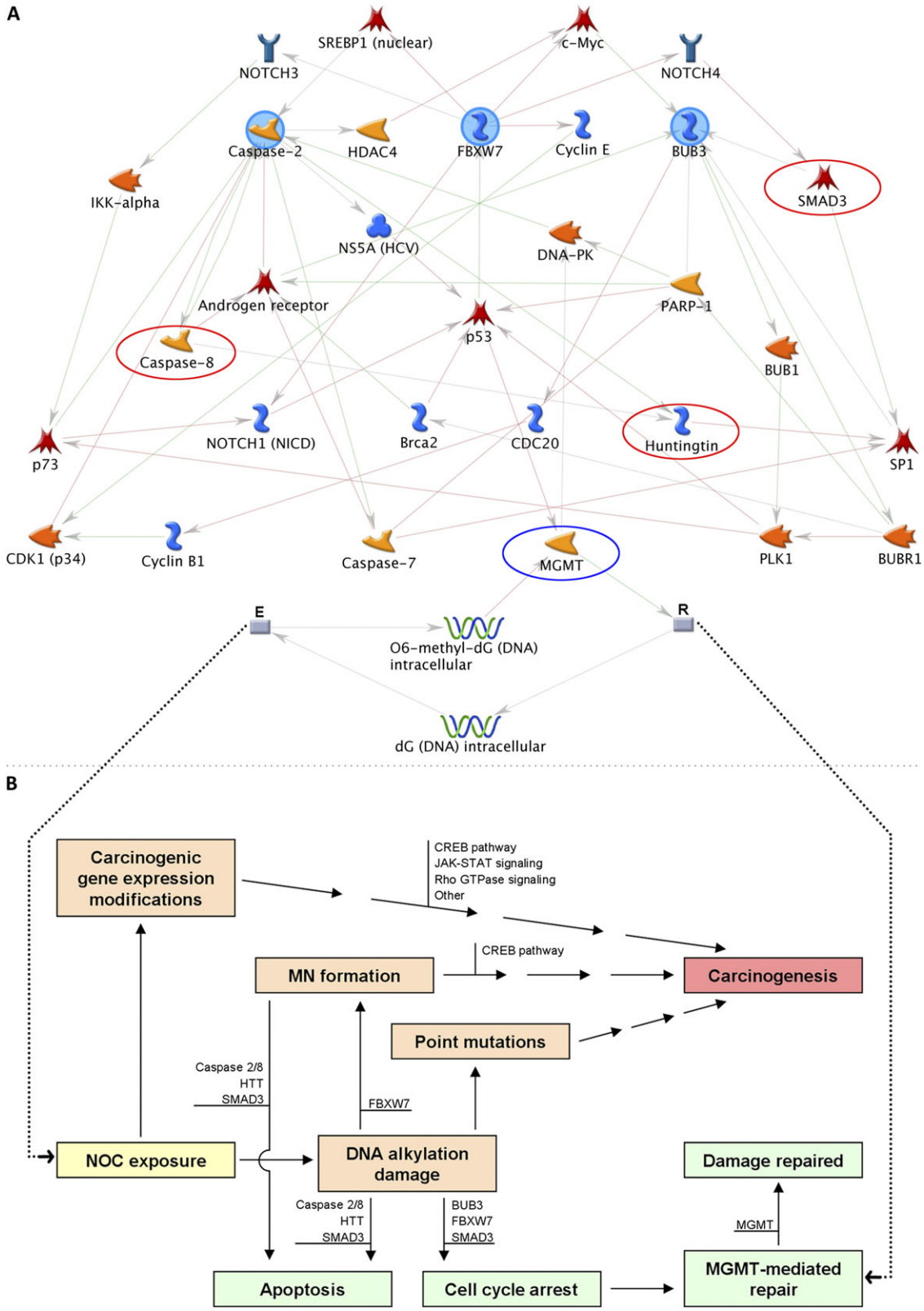


**Fig. 2.** (**A**) Shortest paths network developed in MetaCore based on genes related to FBXW7, BUB3 and caspase 2 shown in blue circles. Ellipses indicate additional genes associated with urinary NOC excretion (in red, i.e. caspase 8, huntingtin and SMAD3) and MN frequency (in blue, i.e. MGMT). Exposure to NOC resulting in DNA damage is indicated by 'E' and the DNA repair process by 'R'. Detailed information on the symbols can be found at http://www.genego.com/pdf/MC_legend.pdf. (**B**) Possible locations are shown for the involvement of the most important genes in the network analysis and some of the modified pathways presented in Tables I–IV, in the route from NOC exposure to repair or other outcomes, such as apoptosis, MN formation and carcinogenesis.

concentrations, which are physiologically more relevant (22). This may be related to a relatively high rate of cytochrome P450 (CYP)-catalysed NOC metabolism in lymphocytes (36). Within this respect, it is interesting to note that apoptosis could also be measured in the CBMN assay in 'cytome' mode (26,37), which may be a valuable addition to future studies.

The analyses also revealed a cell cycle regulation pathway to be associated with NOC excretion (Table II) in addition to several pathways involved in cell adhesion and cytoskeleton remodeling (Tables I and II). These are often linked with the cell cycle regulation and apoptosis process, providing further evidence for NOC-induced genotoxicity since DNA damage, and in particular MN formation, affects the progression of the cell cycle (38). The modifications in apoptosis- and cell cycle-related pathways associated with NOC excretion, in addition to the relation found with MN frequency, indicate that NOC exposure levels found in humans at daily life conditions may contribute to the carcinogenic process.

A relatively large number of pathways appear involved in development and G-protein signaling processes. These pathways are of interest since they involve signaling cascades that are important in the regulation of differentiation and cell proliferation. Signaling pathways that were found to be modulated were implicated in the JAK-STAT signaling cascade and epidermal growth factor receptor (EGFR) signaling and regulation of a number of Rho GTPases. All these pathways play important roles in cell growth, differentiation, cell death, DNA synthesis and cell proliferation, which are crucial in cancer cell development (39–41). These modifications may also be relevant with regard to the NewGeneris objective of identifying prenatal exposures that possibly influence the development of childhood cancer, especially since NDMA can cross the placental barrier, thereby leading to fetal exposure (16,42). In relation to this, the pathways belonging to neurophysiological processes could be important in neural tube defects that arise during fetal development since this has been associated with NOC exposure (43). Other pathways related to NOC excretion include a number of amino acid metabolism pathways, which can probably be attributed to translation-associated protein synthesis, and two immune response pathways that are indicative of immunomodulation and could be relevant in light of the immunosuppressive effects described for NOCs in rodents (44).

Similar tertile and correlation analyses on gene expressions changes and in relation to MN frequencies were performed to investigate whether MN frequency itself was associated with pathways of interest. In the tertile comparison analysis (Table III), only pathways belonging to two cellular processes, i.e. carbohydrate and lipid metabolism, were identified. Most of these pathways were also identified in the correlation analysis (Table IV) and they may be related to three other modified pathways that are more easily interpreted with regard to MN levels, which are the cytoskeleton remodeling pathway describing the role of PKA, the PKA signal transduction pathway itself and the CREB transcription pathway. PKA is dependent on the level of cAMP in the cell, and both cAMP and PKA have been implicated in MN formation in blood cells (45). Moreover, PKA signaling plays an important role in the regulation of glycogen, glucose and lipid metabolism with CREB being an important downstream transcription factor target of PKA (46).

A few developmental pathways involving interleukin 8 (IL-8), endothelin receptor type A (EDNRA) and sphingosine-1-phosphate receptor 3 (EDG3) were also identified and are of special interest since they are all involved in angiogenesis (47–49). The relevance of the transport pathways involving the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a chloride channel and controls the regulation of transport pathways, is difficult to assess.

It is interesting that two of the three overlapping pathways between NOC excretion and MN frequency are associated with PKA and CREB signaling, which are implicated in MN formation as discussed above, and may therefore represent an intermediate mechanism in the link between NOC exposure and MN formation. The continuum between exposure markers, effect markers and disease and the identification of marker-associated transcriptomic responses presents the possibility of using gene expression modifications as mechanistic biomarkers, especially if similar transcriptomic modifications are found in the disease. This constitutes the Meet-in-the-Middle concept as proposed by Vineis and Perera (50). It is therefore especially interesting that the CREB pathway is now being recognised as an important player in human cancer (51).

Recently, we published a transcriptomic network analysis of MN formation-related genes based on available peer-reviewed literature (31). In the present study, the genes included in this previously published network were screened for matches with both the NOC excretion- and the MN frequency-associated expression sets. Three genes were found to match and a network dedicated to find the shortest paths between these genes was created (Figure 2A). As could be expected, FBXW7, which was identified in the MN frequency related gene set, is an important regulator in cell cycle and DNA damage processes but also in aneuploidy and NOTCH signaling (Table V) which is intimately linked with MN formation (52). BUB3 and caspase 2, which were both identified in the NOC excretion related gene set, are mostly involved in cell cycle regulation and apoptosis and DNA damage, respectively. More specifically, BUB3 is an essential

**Table V.** Biological processes regulated by FBXW7, BUB3 and caspase 2

| Genes in MN frequency-related gene set | Genes in total urinary NOC excretion-related gene set | |
|---|---|---|
| FBXW7 | BUB3 | Caspase 2 |
| Aneuploidy | Cell cycle | Apoptosis and survival |
| Cell cycle | Mitotic cell cycle checkpoint | DNA damage |
| DNA damage | Spindle assembly checkpoint | Neoplasm/carcinoma formation |
| Neoplasm/carcinoma formation | | Proteolysis |
| NOTCH signaling | | |
| Proteolysis | | |

Information on genes was obtained from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

component of the spindle checkpoint pathway and defects in BUB genes are known to result in increased MN formation (53). Within this respect, the negative correlation of BUB3 with NOC excretion levels supports the association between NOC excretion and MN formation. FBXW7 and caspase 2 are also implicated in the neoplastic process, stressing their importance in relation to MN formation-associated carcinogenic risk. Although FBXW7, BUB3 and caspase 2 are not directly linked with each other in the network, they have many upstream and downstream genes in common all of which are important in the processes described in Table V. The network was subsequently screened for additional matches with the gene sets used in this study and three more genes (caspase 8, HTT and SMAD3) were found to be present in the gene set correlating with NOC excretion. All three genes had direct interactions with BUB3 or caspase 2 (Figure 2A) and were involved in processes presented in Table V, particularly apoptosis, cell cycle, DNA damage and neoplasm/carcinoma formation. MGMT was added separately to this network since it is one of the most important DNA repair enzymes involved in repairing alkyl adducts and because it was found to be significantly up regulated in the MN frequency tertile analysis. As could be expected, MGMT interacts with the genes already present, including p53. The network and these seven genes in particular are thus likely to influence each other in response to NOC exposure and/or MN formation. The network as such presents a transcriptomic interactome that integrates gene expression modifications in response to NOC exposure, MGMT-mediated repair and MN formation. An overview of where these genes and some of the modified pathways could be implicated in the route from exposure to disease is presented in Figure 2B.

Since this is one of the first studies focused on identifying gene expression modifications in surrogate tissue, further research is needed to validate these results on a larger scale and possibly add other relevant genes or processes to the list. This could be done by screening large cohorts for similar expression profiles of these specific genes in relation to MN frequency and NOC exposure. It may be recommended to screen the gene expression profile in lymphocytes alone, instead of whole blood. Although the basal gene expression profiles of lymphocytes and whole blood is known to show considerable overlap, the presence of reticulocytes in whole blood introduces more variation, causing small gene expression changes to be missed (54). However, whole blood does have the advantage of immediate RNA stabilisation after blood withdrawal, in contrast to the time-consuming procedure required for lymphocyte isolation. Future validation studies might also benefit from measuring NOC-induced DNA adducts in relation to MN frequency and using the CBMN assay in cytome mode to associate other effect markers with NOC exposure, such as MN with/without centromere signals, nucleoplasmic bridges, nuclear buds, cell death (necrosis or apoptosis) and nuclear division rate (26,37). The effects that we observed in whole blood may be of relevance for molecular events in target tissues exposed to NOCs, such as the gastro-intestinal tract or the liver, which would normally be difficult to investigate due to their inaccessibility. Although it is important to note that extrapolation of results found in surrogate tissues to target tissues should be done with caution, blood transcriptomics are known to reflect effects induced in target tissue (55).

In summary, we have for the first time found a link between urinary excretion of NOCs by individuals under daily life circumstances and lymphocytic MN frequency as a marker of effect. Gene expression changes associated with both markers revealed modifications in pathways, which indicate molecular responses related to genotoxicity and carcinogenicity. Our data support a possible role of NOC exposure, through the diet or as a result of endogenous nitrosation processes, in the development of human cancer. Furthermore, the genetic processes and genes found to be modified in this study may be of relevance in future investigations into NOC-associated carcinogenicity in humans.

## Funding

EU Integrated Project NewGeneris, 6th Framework Programme, Priority 5: Food Quality and Safety. NewGeneris is the acronym of the project 'Newborns and Genotoxic exposure risks' (FOOD-CT-2005-016320).

## Acknowledgements

Conflict of interest statement: None declared.

## References

1. Druckrey, H., Preussmann, R., Ivankovic, S. *et al.* (1967) Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats. *Z. Krebsforsch.*, **69**, 103–201.
2. IARC (2010) *Agents Classified by the IARC Monographs, Volumes 1-100*. http://monographs.iarc.fr/ENG/Classification/ClassificationsGroupOrder.pdf (accessed February 5, 2011).
3. Scanlan, R. A. and Barbour, J. F. (1991) N-nitrosodimethylamine content of US and Canadian beers. *IARC Sci. Publ.*, **105**, 242–243.
4. Kuhnle, G. G. and Bingham, S. A. (2007) Dietary meat, endogenous nitrosation and colorectal cancer. *Biochem. Soc. Trans.*, **35**, 1355–1357.
5. Tricker, A. R. (1997) N-nitroso compounds and man: sources of exposure, endogenous formation and occurrence in body fluids. *Eur. J. Cancer Prev.*, **6**, 226–268.
6. Rao, T. K., Lijinsky, W. and Epler, J. L. (eds.) (1984) *Genotoxicology of N-Nitroso Compounds*. Plenum Press, New York, NY.
7. Brambilla, G., Cavanna, M., Pino, A. *et al.* (1981) Quantitative correlation among DNA damaging potency of six N-nitroso compounds and their potency in inducing tumor growth an bacterial mutations. *Carcinogenesis*, **2**, 425–429.
8. Knekt, P., Jarvinen, R., Dich, J. *et al.* (1999) Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. *Int. J. Cancer*, **80**, 852–856.
9. Mirvish, S. S. (1995) Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett.*, **93**, 17–48.
10. de Roos, A. J., Ward, M. H., Lynch, C. F. *et al.* (2003) Nitrate in public water supplies and the risk of colon and rectum cancers. *Epidemiology*, **14**, 640–649.
11. Jakszyn, P., Bingham, S., Pera, G. *et al.* (2006) Endogenous versus exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. *Carcinogenesis*, **27**, 1497–1501.
12. Hebels, D. G., Jennen, D. G., Kleinjans, J. C. *et al.* (2009) Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis. *Toxicol. Sci.*, **108**, 290–300.
13. van Leeuwen, D. M., Pedersen, M., Hendriksen, P. J. *et al.* (2008) Genomic analysis suggests higher susceptibility of children to air pollution. *Carcinogenesis*, **29**, 977–983.
14. van Leeuwen, D. M., Gottschalk, R. W., Schoeters, G. *et al.* (2008) Transcriptome analysis in peripheral blood of humans exposed to environmental carcinogens: a promising new biomarker in environmental health studies. *Environ. Health Perspect.*, **116**, 1519–1525.
15. Mostafa, M. H., Helmi, S., Badawi, A. F. *et al.* (1994) Nitrate, nitrite and volatile N-nitroso compounds in the urine of Schistosoma haematobium and Schistosoma mansoni infected patients. *Carcinogenesis*, **15**, 619–625.
16. Merlo, D. F., Wild, C. P., Kogevinas, M. *et al.* (2009) NewGeneris: a European study on maternal diet during pregnancy and child health. *Cancer Epidemiol. Biomarkers Prev.*, **18**, 5–10.

17. Pedersen, M., Wichmann, J., Autrup, H. *et al.* (2009) Increased micronuclei and bulky DNA adducts in cord blood after maternal exposures to traffic-related air pollution. *Environ. Res.*, **109**, 1012–1020.
18. Lijinsky, W. (1992) *Chemistry and Biology of N-Nitroso Compounds*. Cambridge Univ. Press, Cambridge, UK.
19. Mirvish, S. S., Haorah, J., Zhou, L. *et al.* (2003) N-nitroso compounds in the gastrointestinal tract of rats and in the feces of mice with induced colitis or fed hot dogs or beef. *Carcinogenesis*, **24**, 595–603.
20. Spiegelhalder, B., Eisenbrand, G. and Preussmann, R. (1980) Volatile nitrosamines in food. *Oncology*, **37**, 211–216.
21. Bonassi, S., Znaor, A., Ceppi, M. *et al.* (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, **28**, 625–631.
22. El-Zein, R. A., Fenech, M., Lopez, M. S. *et al.* (2008) Cytokinesis-blocked micronucleus cytome assay biomarkers identify lung cancer cases amongst smokers. *Cancer Epidemiol. Biomarkers Prev.*, **17**, 1111–1119.
23. Mehta, R., Silinskas, K. C., Zucker, P. F. *et al.* (1987) Micronucleus formation induced in rat liver and esophagus by nitrosamines. *Cancer Lett.*, **35**, 313–320.
24. Vermeer, I. T., Pachen, D. M., Dallinga, J. W. *et al.* (1998) Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. *Environ. Health Perspect.*, **106**, 459–463.
25. Decordier, I., Papine, A., Plas, G. *et al.* (2009) Automated image analysis of cytokinesis-blocked micronuclei: an adapted protocol and a validated scoring procedure for biomonitoring. *Mutagenesis*, **24**, 85–93.
26. Kirsch-Volders, M., Plas, G., Elhajouji, A. *et al.* (2011) The *in vitro* MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance. *Arch. Toxicol.*, Doi:10.1007/s00204-011-0691-4.
27. Fenech, M., Chang, W. P., Kirsch-Volders, M. *et al.* (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res.*, **534**, 65–75.
28. Chen, X., Ender, P., Mitchell, M. *et al.* (2003) *SPSS Web Books*. http://www.ats.ucla.edu/stat/spss/webbooks/reg/default.htm (accessed February 26, 2011).
29. Shi, L., Reid, L. H., Jones, W. D. *et al.* (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat. Biotechnol.*, **24**, 1151–1161.
30. Ekins, S., Nikolsky, Y., Bugrim, A. *et al.* (2007) Pathway mapping tools for analysis of high content data. *Methods Mol. Biol.*, **356**, 319–350.
31. van Leeuwen, D. M., Pedersen, M., Knudsen, L. E. *et al.* (2011) Transcriptomic network analysis of micronuclei-related genes: a case study. *Mutagenesis*, **26**, 27–32.
32. Georgiadis, P., Samoli, E., Kaila, S. *et al.* (2000) Ubiquitous presence of O6-methylguanine in human peripheral and cord blood DNA. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 299–305.
33. Speit, G., Zeller, J. and Neuss, S. (2011) The in vivo or ex vivo origin of micronuclei measured in human biomonitoring studies. *Mutagenesis*, **26**, 107–110.
34. Arranz, N., Haza, A. I., Garcia, A. *et al.* (2008) Inhibition by vitamin C of apoptosis induced by N-nitrosamines in HepG2 and HL-60 cells. *J. Appl. Toxicol.*, **28**, 788–796.
35. Uhl, M., Helma, C. and Knasmuller, S. (1999) Single-cell gel electrophoresis assays with human-derived hepatoma (Hep G2) cells. *Mutat. Res.*, **441**, 215–224.
36. Raucy, J. L., Schultz, E. D., Wester, M. R. *et al.* (1997) Human lymphocyte cytochrome P450 2E1, a putative marker for alcohol-mediated changes in hepatic chlorzoxazone activity. *Drug Metab. Dispos.*, **25**, 1429–1435.
37. Thomas, P. and Fenech, M. (2011) Cytokinesis-block micronucleus cytome assay in lymphocytes. *Methods Mol. Biol.*, **682**, 217–234.
38. Kim, B. M., Choi, J. Y., Kim, Y. J. *et al.* (2007) Reoxygenation following hypoxia activates DNA-damage checkpoint signaling pathways that suppress cell-cycle progression in cultured human lymphocytes. *FEBS Lett.*, **581**, 3005–3012.
39. Aaronson, D. S. and Horvath, C. M. (2002) A road map for those who don't know JAK-STAT. *Science*, **296**, 1653–1655.
40. Bustelo, X. R., Sauzeau, V. and Berenjeno, I. M. (2007) GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays*, **29**, 356–370.
41. Oda, K., Matsuoka, Y., Funahashi, A. *et al.* (2005) A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol. Syst. Biol.*, **1**, 2005 0010.
42. Annola, K., Heikkinen, A. T., Partanen, H. *et al.* (2009) Transplacental transfer of nitrosodimethylamine in perfused human placenta. *Placenta*, **30**, 277–283.
43. Ward, M. H., deKok, T. M., Levallois, P. *et al.* (2005) Workgroup report: drinking-water nitrate and health—recent findings and research needs. *Environ. Health Perspect.*, **113**, 1607–1614.
44. Haggerty, H. G. and Holsapple, M. P. (1990) Role of metabolism in dimethylnitrosamine-induced immunosuppression: a review. *Toxicology*, **63**, 1–23.
45. Suzuki, Y., Takagi, R., Kawasaki, I. *et al.* (2008) The micronucleus test and erythropoiesis: effects of cyclic adenosine monophosphate (cAMP) on micronucleus formation. *Mutat. Res.*, **655**, 47–51.
46. Herzig, S., Hedrick, S., Morantte, I. *et al.* (2003) CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. *Nature*, **426**, 190–193.
47. Li, A., Dubey, S., Varney, M. L. *et al.* (2003) IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J. Immunol.*, **170**, 3369–3376.
48. Smollich, M. and Wulfing, P. (2008) Targeting the endothelin system: novel therapeutic options in gynecological, urological and breast cancers. *Expert Rev, Anticancer Ther.*, **8**, 1481–1493.
49. Waeber, C., Blondeau, N. and Salomone, S. (2004) Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. *Drug News Perspect.*, **17**, 365–382.
50. Vineis, P. and Perera, F. (2007) Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 1954–1965.
51. Conkright, M. D. and Montminy, M. (2005) CREB: the unindicted cancer co-conspirator. *Trends Cell Biol.*, **15**, 457–459.
52. Curry, C. L., Reed, L. L., Broude, E. *et al.* (2007) Notch inhibition in Kaposi's sarcoma tumor cells leads to mitotic catastrophe through nuclear factor-kappaB signaling. *Mol. Cancer Ther.*, **6**, 1983–1992.
53. Kalitsis, P., Earle, E., Fowler, K. J. *et al.* (2000) Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev.*, **14**, 2277–2282.
54. Raghavachari, N., Xu, X., Munson, P. J. *et al.* (2009) Characterization of whole blood gene expression profiles as a sequel to globin mRNA reduction in patients with sickle cell disease. *PLoS One*, **4**, e6484.
55. Huang, J., Shi, W., Zhang, J. *et al.* (2010) Genomic indicators in the blood predict drug-induced liver injury. *Pharmacogenomics J*, **10**, 267–277.