Micronuclei in Cord Blood Lymphocytes and Associations with Biomarkers of Exposure to Carcinogens and Hormonally Active Factors, Gene Polymorphisms, and Gene Expression: The NewGeneris Cohort

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Micronuclei in Cord Blood Lymphocytes and Associations with Biomarkers of Exposure to Carcinogens and Hormonally Active Factors, Gene Polymorphisms, and Gene Expression: The NewGeneris Cohort

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BACKGROUND: Leukemia incidence has increased in recent decades among European children, suggesting that early-life environmental exposures play an important role in disease development.

OBJECTIVES: We investigated the hypothesis that childhood susceptibility may increase as a result of in utero exposure to carcinogens and hormonally active factors. Using cord blood samples from the NewGeneris cohort, we examined associations between a range of biomarkers of carcinogen exposure and hormonally active factors with micronuclei (MN) frequency as a proxy measure of cancer risk. Associations with gene expression and genotype were also explored.

METHODS: DNA and protein adducts, gene expression profiles, circulating hormonally active factors, and GWAS (genome-wide association study) data were investigated in relation to genomic damage measured by MN frequency in lymphocytes from 623 newborns enrolled between 2006 and 2010 across Europe.

RESULTS: Malondialdehyde DNA adducts (M_{d}G) were associated with increased MN frequency in biunucleated lymphocytes (MBN), and exposure to androgenic, estrogenic, and dioxin-like compounds was associated with MN frequency in mononucleated lymphocytes (MMN), although no monotonic exposure-outcome relationship was observed. Lower frequencies of MBN were associated with a 1-unit increase expression of PDCD11, LAT2, TRIM13, CD28, SMC1A, IL7R, and NIPBL genes. Gene expression was significantly higher in association with the highest versus lowest category of bulky and M_{d}G–DNA adducts for five and six genes, respectively. Gene expression levels were significantly lower for 11 genes in association with the highest versus lowest category of plasma AR CALUX (chemically activated luciferase expression for androgens) (8 genes), ERO CALUX (for estrogens) (2 genes), and DR CALUX (for dioxins). Several SNPs (single-nucleotide polymorphisms) on chromosome 11 near FOLLI significantly modified associations between androgen activity and MBN frequency. Polymorphisms in EPHX12 and CYP2E1 were associated with MBN.

CONCLUSION: We measured in utero exposure to selected environmental carcinogens and circulating hormonally active factors and detected associations with MN frequency in newborns circulating T lymphocytes. The results highlight mechanisms that may contribute to carcinogen-induced leukemia and require further research.

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Introduction

Cancer incidence among European children, specifically leukemia, has steadily increased over the last three decades (Kaatsch 2010). In view of the relatively short latency period for leukemia and its very early onset in childhood, it has been suggested that fetal exposure to environmental carcinogens may increase susceptibility to this cancer (Wild and Kleinjans 2003).

The European Union (EU)–funded project Newborns and Genotoxic exposure risks (NewGeneris) was designed to evaluate the hypothesis that maternal intake of dietary and other environmental carcinogens results in in utero exposure and early biological effects in the unborn child, possibly leading to increased risk of cancer in later childhood (Merlo et al. 2009). The primary aim of the present study was to investigate the
Materials and Methods

Study population and sample collection. Pregnant women (n = 1,200) were enrolled between 2006 and 2010 in Heraklion, Crete, Greece; Barcelona and Sabadell, Spain; Bradford, England; Copenhagen, Denmark; and Oslo and Akershus, Norway (Pedersen et al. 2012). The participation of mothers in the study was based on previously described eligibility criteria (Pedersen et al. 2012). Study protocols were approved by local ethics committees, and informed consent was obtained from all participating mothers before sample collection.

Detailed information on personal characteristics, including demographic, health, and lifestyle factors, was obtained using extensive questionnaires completed by mothers before or around the time of the delivery. Information on dietary habits during pregnancy was obtained from maternity records. Gestational age (completed weeks) was computed based on last menstrual period or ultrasound-based estimated date of conception.

Blood samples were collected from 1,151 mother–infant dyads following a common protocol as described previously (Merlo et al. 2009). Umbilical cord blood samples were collected immediately after birth from the cord vein of newborns and locally processed. Samples were kept at –20°C or –80°C until shipment on dry ice to the study laboratories.

Biomarkers of exposure and early biological effect. DR CALUX® bioassay. Dioxin-like activity, expressed as aryl hydrocarbon receptor (AhR)–mediated activation of the chemically activated luciferase gene expression (CALUX®) bioassay were used to assess exposure to androgenic (AR), estrogenic (ERα), and dioxin-like (DR) compounds.

Facilitated by the development of microarray technologies, gene expression–based biomarkers have been developed and applied for human biomonitoring purposes (McHale et al. 2011; Rager et al. 2011; Ren et al. 2011; van Leeuwen et al. 2008). Gene expression profiling has the potential to identify new biomarkers of exposure that may simultaneously reflect the earliest biological events in disease pathogenesis. Here, we evaluated the expression of 36 genes that were associated with biomarkers of carcinogenic exposure by quantitative real-time polymerase chain reaction (qRT-PCR) (Hochstenbach et al. 2012).

MN frequency was assessed as the primary outcome. MN are a potential biomarker of cancer risk, because increased micronucleated binucleated (MNBN) frequencies in T lymphocytes have been shown to be associated with cancer risk in adults (Bonassi et al. 2007). MN are small extranuclear bodies arising in dividing cells that are caused by chromosomal breakage and/or whole chromosome loss (Fenech 2007; Kirsch-Volders et al. 2011). MNBN provide a measure of the lesions that have recently occurred in vivo, whereas micronucleated mononucleated lymphocytes (MNMONO) give an estimation of the genome damage accumulated over a long period in stem cells and circulating lymphocytes (Kirsch-Volders and Fenech 2001).

Furthermore, we performed a genome-wide association study (GWAS) to investigate whether associations between exposure biomarkers and MN are modified by genetic variation.

Hb adducts. Erythrocytes were isolated by centrifugation on the day of collection and stored at –20°C. AA-, GA-, and EtO–Hb adducts were simultaneously determined by the adduct FIRE procedure using liquid chromatography tandem mass spectrometry with performance and validation standards as described in detail elsewhere (von Stedingk et al. 2010, 2011). In total, Hb adduct levels were measured in 1,151 cord blood samples.

DNA adducts. DNA was isolated with the Qiagen Midi Kit (no. 13343; Qiagen, Hilden, Germany) with some modifications of the manufacturer’s protocol, as reported previously (Kovács et al. 2011). Additional details are provided in Supplemental Material (pp. 4–8).

Immunoblot analysis of M1,dG. M1,dG was determined by an immunoblot blot method, using a murine M1,dG monoclonal primary antibody (D10A1), provided by L. Marnett (Vanderbilt University, TN, USA), as described previously (Singh et al. 2001).

Immunochemoassays for analyses of O6-MedG and PAH–DNA adducts. These analyses were carried out using ultrasensitive sandwich chemiluminescence immunoassays as previously described for O6-MedG (Georgiadis et al. 2011) and PAH (polycyclic aromatic hydrocarbons)–DNA adducts (Georgiadis et al. 2012).

Postlabeling analysis of bulky DNA adducts. Bulky DNA adducts were detected with the nuclease P1 modification of the 32P-postlabeling procedure as described elsewhere (Kovács et al. 2011). Interlaboratory differences in levels were adjusted for, as described in Supplemental Material (p. 8).

Cytokinesis block micronuclear assay. The in vitro cytokinesis blocked MN assay was carried out according to the standardized protocol developed for semiautomated image analysis (Decordier et al. 2009) and adapted for umbilical blood (Vande Loock et al. 2011). MN were scored in both MNBN and MNMONO T lymphocytes (Kirsch-Volders and Fenech 2001). To harmonize slide preparation, the cohort cytologists were trained by I.D., K.V.L., and M.K.-V. (Vrije Universiteit Brussel; VUB). Slides were sent to VUB, where staining and MN analysis occurred. Quality control after staining included visual selection of slides with good quality, using a light microscope and based on a good spreading, swelling, and amount of cells. The automated scoring procedure followed by visual validation of selected micronucleated cells was carried out by the same researcher, using the PathFinder™ platform installed by IMSTAR S.A. (Paris, France) at the VUB laboratory: this consisted of a PathFinder™ CELLSCAN™ capture station and two PathFinder™ MN analysis workstations. Reproducibility of the automated...
Micronuclei, exposure biomarkers, and gene expression in newborns

We conducted a genome-wide scan of approximately 300,000 tagging single nucleotide polymorphisms (SNPs) using the Illumina HumanCytoSNP-12 v1 (Illumina Inc., Hayward, CA, USA) according to the manufacturer’s protocols. Genotype calling was done using Illumina GenomeStudio 2010. Genomic DNA was isolated from 900 cord blood samples and was used to genotype each child. Quality control was performed on a per-sample and per-SNP basis. We excluded 33 duplicates, 23 samples with a genotype call rate < 98.5%, and 14 twins, leaving 830 genotyped samples available for analysis. We used a general genetic model retaining the three distinct genotypes and without making any assumption about the direction of the SNP’s association in the heterozygote compared with the two homozygote classes. According to nonmutually exclusive SNP-based quality checks, 6,801 SNPs were excluded because of Hardy–Weinberg equilibrium violation ($p < 10^{-6}$), 35,429 because they had a minor allele frequency (MAF) < 1%, and 7,338 because missing genotype was > 10%, resulting in 258,246 of 298,199 SNPs left for statistical analyses. A total of 435 newborns had both SNPs and MN results available, and they were used in GWAS statistical analyses.

In addition, SNPs present in metabolism and DNA repair genes were selected a priori by the consortium as candidate genes based on the available knowledge on functionalities with respect to bioactivation (CYP1A1, CYP2E1, CYP2D6, EPHX1, and EPHX2) and detoxification (GSTM1) of DNA adduct-forming metabolites, base excision repair of oxidative adducts (OGG1), nucleotide excision repair of bulky adducts (XRCC1, ERCC2/XP, XP, and XRCC3), repair of alkylated adducts (MGMT, ALKB, and MPG) and of thymine adducts (TDG), and with respect to folate metabolism, which is known to interfere with micronucleus formation (MTHFR, MTR, and MTRR).

**Statistical analyses.** Separate negative binomial fixed effects multivariable regression models were used to estimate the associations of MN frequencies per 1,000 MNBN with exposure biomarkers. The parameter of interest, adjusted for covariates, was the log odds ratio (OR) of the strength of association between the exposure category and the associations between biomarkers and SNPs. We estimated associations between the expression of each of the 36 genes evaluated and categorical exposure biomarkers using separate multivariable linear regression models adjusted for the covariates listed above. The F-test was used as a global test of statistical significance over all categories of each exposure biomarker. SNPs were selected a priori, and the interactions between exposure biomarkers with gene expression and with SNPs were assessed. We estimated associations between the expression of each of the 36 genes evaluated and categorical exposure biomarkers using separate multivariable linear regression models adjusted for the covariates listed above. The F-test was used as a global test of statistical significance over all categories of each exposure biomarker. For each exposure biomarker we report the differences in gene expression associated with the highest versus lowest category of exposure biomarkers.

For gene expression and GWAS analyses, we adjusted the estimated p-values to account for multiple comparisons using standard methods (Benjamini and Hochberg 1995; Hochberg 1988; Holm 1979). This criterion was used to identify SNPs associated with MN as main predictors or as effect modifiers of the exposure biomarkers–MN and gene expression–MN associations. No adjustment was made for p-values estimated from the analyses of a priori–selected candidate genes. p-Values < 0.05 were considered statistically significant. All associations were examined in newborns with MN assay data available ($n = 623$) and with exposure biomarkers, gene expression, and GWAS data available. Sample sizes for individual association analyses varied as indicated in the results.

Manhattan plots for p-values along chromosomes and position were made by the genetic analysis package (gap) for CRAN R 2.11.2. Statistical analyses were carried out using Stata S.E. version 10.0 (StataCorp, College Station, TX, USA), R (http://cran.r-project.org), and Genedata Expressionist 7.0 (Genedata AG, Basel, Switzerland).
Results

Levels of biomarkers of exposure (i.e., Hb and DNA adducts, and AR, ERα, and DR CALUX® activity) detected in newborns are reported in Table 1. The number of observations for each biomarker varied reflecting the variable amount of biological specimens collected from cord blood and the assays prioritized adopted (i.e., Hb adducts, DNA adducts, and CALUX® activity). The largest number of observations was available for AA–Hb adducts (n = 1,151) and the smallest for DR CALUX® (n = 725). For all biomarkers large variations were present (e.g., AA–Hb adducts: median = 14.4 pmol/g Hb; range, 4.4–124.8; M1dG–DNA adducts: median = 9.9/10⁶ nucleotides; range, 0.5–324.7).

Descriptive statistics for MNBN and MNMONO T lymphocytes are shown in Table 2 by cohort and by sociodemographic, reproductive, and lifestyle factors. Again large interindividual variations were observed within and between cohorts, with the highest level of MN observed in Greece (MNBN mean = 1.79 ± 1.50 per 1,000 binucleated T lymphocytes) and the lowest in the United Kingdom (MNBN mean = 0.55 ± 0.74).

None of the global tests of associations across all categories of exposure were statistically significant, and there was no evidence of monotonic dose–response trends with increasing levels of exposure for associations of AA–, GA–, or EtO–Hb adducts (quintiles); PAH–, bulky–, or O6-MG–DNA adducts (quartiles); or DR CALUX® plasma levels (quartiles) and frequencies of MNBN and MNMONO T lymphocytes (Table 3). A significant overall association was found between M1dG levels and the frequency of MNBN lymphocytes, although associations relative to the lowest quartile of M1dG were positive for the second and third quartiles and negative for the highest quartile. ERα CALUX® plasma levels were significantly associated with the frequency of MNBN and MNMONO lymphocytes and AR CALUX® with the frequency of MNMONO lymphocytes. No monotonic exposure–outcome association was observed between ERα CALUX® or AR CALUX® and MN. For ERα CALUX® a significant negative association with MNBN was detected for the second quartile, followed by a weak nonsignificant positive association with the third and fourth quartiles while the associations with MNMONO were negative for the second and fourth quartiles. The strongest associations were detected for AR CALUX® and MNMONO T lymphocytes and were positive for the second and third quartiles and negative for the fourth quartile.

One-unit increases in the expression of 7 of the 36 genes evaluated (PDCD11, LAT2, TRIM13, CD28, SMCA1, IL7R, and NIPBL) were associated with significantly lower MNBN frequencies, with MR ranging from 0.81 (95% CI: 0.88, 0.96) for PDCD11 to 0.64 (95% CI: 0.77, 0.97) for NIPBL (Figure 1A). The frequency of MNMONO was not significantly associated with expression of any of the genes tested (data not shown).
Micronuclei, exposure biomarkers, and gene expression in newborns

In models with gene expression levels as the dependent variable, expression was significantly higher in association with the highest versus lowest category of bulky DNA adducts and of M$_{1}$dG levels for five and six genes, respectively (Figure 1B). Conversely, expression levels were significantly lower for a total of 11 genes in association with the highest versus lowest category of plasma AR CALUX$^*$ (eight genes), ERα CALUX$^*$ (two genes), and DR CALUX$^*$ (seven genes) (Figure 1B). Associations with lower levels of exposure are not reported. Six of the seven genes whose expression was associated with significantly lower MNBN frequency (i.e., all except TRIM13; Figure 1A) were significantly associated with the highest versus lowest category of at least one exposure biomarker (M$_{1}$dG, DR CALUX$^*$, ERα CALUX$^*$, or AR CALUX$^*$; Figure 1B).

GWAS was carried out on 435 newborns with data available for both SNPs and micronuclei. Confounding by population stratification was assessed (see Supplemental Material, Figures S1 and S2) and confirmed that genotypetype variations occurred between population subgroups (i.e., maternal ethnicity and newborns’ country of birth), justifying the need for adjustment in statistical analyses. None of the GWAS SNPs were significant predictors of MNBN frequencies (see Supplemental Material, Figure S3). Investigation of the exposure biomarkers–SNPs interactions on the occurrence of MNBN revealed a cluster of significant SNPs (on chromosome 11) for AR CALUX$^*$ modeled as a continuous variable (see Supplemental Material, Figure S4). The four SNPs acting as effect modifiers of the relationship between AR CALUX$^*$ and the frequency of MNBN lymphocytes are given in Supplemental Material, Table S2. The association of these SNPs were reported per unit increase of plasma AR CALUX$^*$ and varied according to the allele variants. For each of the SNPs shown, there was a significant positive association between a 1-unit increase in plasma AR CALUX$^*$ and MNBN frequency among participants with one homozygous genotype, and a significant negative association with the alternate homozygous genotype (e.g., for rs7131537, MR = 2.54; 95% CI: 1.69, 3.75 for CC and MR = 0.36; 95% CI: 0.21, 0.60 for AA, with a null association among AC heterozygotes compared with an overall estimated association MR = 0.36; 95% CI: 0.21, 0.60). A gene SNPs were significantly associated with...
the frequency of MNMONO lymphocytes (data not shown).

Discussion

Here, we show that exposure biomarkers and T lymphocyte MN levels are measurable in cord blood, that large variations exist for these in the European newborn population, and also that some of the exposure biomarkers are associated with MN levels (as independent variables) and with gene polymorphisms (when the biomarkers are modeled as dependent variables). This suggests that the fetus may be exposed to carcinogenic chemicals in utero via the placenta, and that such exposures may be sufficient to exert early biological effects manifested as an increase in the frequency of MNBN, a marker that has been associated with cancer risk in adults (Bonassi et al. 2007). However, our findings should be interpreted with caution given that associations did not show evidence of consistent dose–response relations with increasing levels of exposure.

M1dG is the major DNA adduct arising from malondialdehyde, a genotoxic by-product of lipid peroxidation of polyunsaturated fatty acids with a high number of double bonds that also can be formed during food preparation (Jeong and Swenberg 2005). A significant overall association was detected between M1dG adduct levels and MNBN frequency, although the positive association was limited to the second and third quartiles, with the highest quartile of M1dG adducts being associated with the lowest MNBN frequency when compared with the lowest quartile. This association indicates recent exposure to malondialdehyde, because MNBN formation reflects recent genetic damage that results in micronuclei formation when cell replication is induced in vitro. No association was found between Hb adducts with MNMONO frequencies; however, fetal exposure to compounds detected by ERα CALUX and AR CALUX induced significant increases of MNMONO, possibly reflecting genetic damage accumulated during fetal development (Kirsch-Volders and Fenech 2001). The CALUX assays measure estrogenic, androgenic, or dioxin-like activities that could result from a variety of compounds or mixtures of compounds. Consequently, associations cannot be attributed to specific exposures. Infant acute leukemia is a frequent childhood cancer, and maternal exposure to hormones during pregnancy has been reported as a potential risk in disease occurrence (Pombo-de-Oliveira and Koifman 2006). A recent review (Holland et al. 2011) on MN in neonates and children concluded that exposure to environmental pollutants and radiation leads to increased MN; however, no information was provided on possible associations with other biomarkers of exposure and/or early effect, as presented in the present study.

The reduced number of samples available for the statistical analyses of the relationships between exposure biomarkers and MN levels is a limitation of the study and may have introduced false-negative findings. Conversely, some of the detected significant associations may have resulted from the multiple comparisons performed, increasing the chance of false-positive findings. In addition, none of the observed associations followed a dose–response pattern.

We explored the expression of 36 genes by qRT-PCR as potential new biomarkers of toxic exposure. The expression of seven genes was negatively associated with MNBN (none with MNMONO), namely SMC1A, LAT52, TRIM13, PDCD11, CD28, IL7R, and NIPBL. The expression of these particular genes has previously been shown to be affected by one or more genotoxic carcinogens in experimental models (Mattingly et al. 2003). However, because detailed exposure data were absent, we could not further substantiate the involvement of specific chemicals. Using the dedicated TRANSFAC® software (BIODEBASE Biological Databases, Beverly, MA, USA; http://www.biobase-international.com) for finding transcription factor expression in our transcriptomic data, we identified no transcription factor that could regulate all of these genes. Given that MN are formed during metaphase/anaphase/telephase transition, it}

Figure 1. Associations between gene expression and MNBN T-lymphocyte frequency (A) and between exposure biomarkers and gene expression (B) adjusted for country, maternal age, prepregnancy BMI, birth weight, sex, maternal ethnicity, gestational age, delivery, maternal smoking, and ETS. Associations shown are those with multiple comparisons–adjusted p-values < 0.05. (A) Mean ratios (MR) are for associations between 1-unit increases in gene expression and MNBN based on 350 observations with complete data. (B) Differences in gene expression associated with the highest versus lowest category of exposure biomarkers are based on observations with complete data (bulky DNA adducts n = 398, M1dG DNA adducts n = 533, AR CALUX® n = 457; DR CALUX® n = 477; ER CALUX® n = 457). DR CALUX® categories were based on the following centiles: < 0.13; 0.131–0.23; > 0.23 (pg TEQ/mL plasma) because 46% of the observations were tied (i.e., below the limit of detection of the assay). Boxed genes are significant predictors of MNBN and are significantly predicted by at least one exposure biomarker. Red rectangles are mean ratio point estimates of the differences in gene expression associated with the highest versus lowest category of exposure biomarkers (B). Whiskers are 95% CIs.
was of interest that most of the genes identified are involved in progression through the cell cycle, cell division, spindle formation, or DNA damage responses. *SMCIA* encodes a protein that is part of the cohesin protein complex and is involved in sister chromatid cohesion during the cell cycle (Bauerschmidt et al. 2011). The tumor suppressor gene *LAT52* encodes a protein that interacts with centrosome proteins and is required for correct spindle formation (Abe et al. 2006). *TRIM13* encodes a kinase involved in many different cellular processes including proliferation and apoptosis (Nakashima 2002). Furthermore, *CD28* and *PDCD11A* are involved in apoptosis (Lacana and D’Adamo 1999; Walker et al. 1998). *NIPBL* is required for association of cohesin with chromosomes, for early processing of double-strand breaks and for the DNA damage checkpoint (Oka et al. 2011). For *IL7R*, the biological relevance for its association with MNBN remains unclear.

The expression of six of the seven genes associated with MNBN was also associated with the highest versus lowest level of one or more exposure biomarkers (Figure 1). *CD28*, *IL7R*, and *PDCD11A* were associated with the mutagenic DNA adduct M,DG. *CD28* and *PDCD11A* are mainly involved in processes linked to genotoxic stress, such as apoptosis and cell cycle (Lacana and D’Adamo 1999; Walker et al. 1998). *LAT52* and *SMC1A* were associated with DR CALUX*, through which compounds that activate the transcription factor AhR, such as PCDDs (polychlorinated dibenzodioxins), PCDFs (polychlorinated dibenzo-pfurans), dioxin-like PCBs (polychlorinated biphenyls), and PAHs (Pedersen et al. 2010) are measured; many of the latter are genotoxic. Activation of the AhR participates in pathways such as cell cycle regulation, apoptosis and immune responses (Marlowe and Puga 2005). Although *LAT52* and *SMC1A* are not known to be regulated by AhR, both genes are involved in certain subprocesses of the cell cycle. *NIPBL* was associated with AR CALUX*, which measures compounds with androgenic activity. Like AhR, AR is a transcription factor and regulates the expression of various genes involved in cell cycle control, apoptosis, cell growth, and differentiation (Heisler et al. 1997). Although *NIPBL* is not known to be regulated by AR, it is linked to genotoxic stress related processes and is involved in the cell cycle through its mediating function in sister chromatid cohesion (Watrin et al. 2006).

In summary, associations between gene expression profiles and MN induction reflect the origin of MN: Many of the genes are associated with chromosome breakage or loss, and particularly interference with spindle and chromatid segregation. Their associations with exposure biomarkers support their relevance in relation to genotoxic processes.

The analysis of genetic susceptibility was conducted using GWAS. A strong signal was observed on chromosome 11 for an interaction with AR CALUX* on MNBN frequency (see Supplemental Material, Table S2, Figure S4). The gene closest to this hotspot is *FOLH1* (folate hydrolase 1) and could thus be the genetic factor that affects this relationship. Several pseudogenes were closer, but were excluded because their function is unclear. *FOLH1*, also known as PMSA (prostate-specific membrane antigen), is overexpressed in prostate cancer and is negatively regulated by androgen (Ghosh et al. 2005). Furthermore, a polymorphism in *FOLH1* associated with lower levels of serum folate and hyperhomocysteinemia has been described (Devlin et al. 2000). Low folate is recognized as a risk factor for chromosome instability (Ames 2001) and MN induction (Fench and Crott 2002). An interaction between androgen exposure and a polymorphism that modulates *FOLH1* expression might affect folate levels and thereby modify MNBN frequencies.

GWAS was carried out on 435 newborns with data available for both SNPs and micronuclei. The relatively small sample size is a limitation of the GWAS analysis and is likely to have introduced a risk of false-negative findings due to reduced statistical power to detect the studied associations. To reduce false-positive findings, we accounted for multiple comparisons in our primary GWAS analysis, although candidate gene analyses were not adjusted for multiple comparisons. We identified significant associations between a priori-selected SNPs in *EPHX1, EPHX2*, and *CYP2E1* and the frequency of MNBN lymphocytes (Table 4). These SNPs do not affect the protein code, but might be in linkage disequilibrium with causative variants. However, noncausal associations cannot be ruled out, and further clarification is required given inconsistent associations reported between these genes and MN in the literature (Dhillon et al. 2011).

In this study, samples from almost 1,200 newborns were collected. Because of limited sample volumes, the number of biomarker measurements varied from 1,151 for the AA–Hb adduct to 623 for MNBN, and 435 newborns had data available for both SNPs and micronuclei. For some analyses data were available for a limited number of observations: between 434 and 424 subjects for the associations between MNBN and candidate SNPs, and <220 subjects for the interactions SNPs–exposure biomarkers on MNBN frequency. Although we were able to conduct association studies between individual exposure markers with MNBN, this seriously limited our ability to investigate the interaction between multiple exposure biomarkers and MNBN.

### Table 4. Relationships between the available SNPs for the a priori–selected *EPHX* and *CYP2E1* genes and frequency of MNBN T lymphocytes in newborns.

| SNP     | Gene   | n   | MNBN* (mean ± SE) | MR (95 CI)% | p-Value* |
|---------|--------|-----|-------------------|-------------|----------|
| Rs1051741 | EPHX1 | 424 | 1.20 ± 0.07       | 1           | 0.011    |
|         |        |     | 0.12 ± 0.11       | 1.04 (0.85, 1.27) |
|         |        |     | 0.32 ± 0.15       | 0.35 (0.17, 0.71) |
| Rs4148244 | EPHX2 | 434 | 1.17 ± 0.06       | 1           | 0.032    |
|         |        |     | 1.04 ± 0.16       | 0.85 (0.66, 1.08) |
|         |        |     | 1.86 ± 0.48       | 1.89 (1.05, 3.39) |
| Rs2480258/Rs915906 | CYP2E1 | 347 | 1.03 ± 0.06       | 1           | 0.040    |
|         |        |     | 1.14 ± 0.33       | 1.53 (1.02, 2.28) |
| Rs2480258 | CYP2E1 | 347 | 1.07 ± 0.08       | 1           | 0.268    |
|         |        |     | 1.05 ± 0.10       | 1.02 (0.86, 1.22) |
|         |        |     | 1.06 ± 0.25       | 1.38 (0.96, 1.90) |
| Rs915906 | CYP2E1 | 347 | 1.04 ± 0.08       | 1           | 0.265    |
|         |        |     | 1.01 ± 0.10       | 1.07 (0.88, 1.29) |
|         |        |     | 1.09 ± 0.32       | 1.50 (0.93, 2.24) |

Analyses carried out on 18 candidate genes with 89 SNPs available on the array. Only statistically significant relationships are reported. For the Rs2480258/Rs915906 SNPs, relationships estimated for the single SNPs are shown.

*p-Mean ± SE per 1,000 binucleated T lymphocytes. *Mean ratio adjusted for country, maternal age, pregnancy BMI, birth weight, sex, maternal ethnicity, gestational age, delivery, maternal smoking, and ETS. *Log-likelihood ratio test, unadjusted for multiple comparisons.
generate new hypotheses about mechanisms of carcinogen-induced leukemias. The associations that we report must be interpreted with caution because we did not measure specific exposures, we did not observe monotonic dose–response relations, and we cannot rule out noncausal associations.

Nevertheless, our results suggest that internal exposure of the fetus to toxic chemicals occurs during apparently normal pregnancies, that such exposures may increase the frequency of MN formation [which, although of uncertain relevance in newborns (Holland et al. 2011) has been associated with cancer risk in adults (Bonassi et al. 2007)], and that some children may be more susceptible to genotoxic effects of in utero exposures than others.

Ultimately, information on the effects and sources of in utero genotoxic exposures could be used by regulators and industry to develop policy measures and strategies to reduce such exposures in order to improve children’s health and reduce the incidence of childhood cancer.

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