Global gene expression analysis in cord blood reveals gender-specific differences in response to carcinogenic exposure *in utero*

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Abstract

Background: It has been suggested that such foetal exposure might lead to predisposition to develop cancer during childhood or in later life possibly through modulation of the foetal transcriptome. Since gender effects in the incidence of childhood cancers have been described, we hypothesized differences at the transcriptomic level in cord blood between male and female newborns as a consequence of foetal carcinogenic exposure. The objective was to investigate whether transcriptomic responses to dietary genotoxic and non-genotoxic carcinogens demonstrate gender-specific mechanisms-of-action relevant for chemical carcinogenesis.

Methods: Global gene expression was applied in umbilical cord blood samples, the CALUX®-assay was used for measuring dioxin(-like), androgen(-like) and estrogen(-like) internal exposure, and acrylamide-haemoglobin adduct levels were determined by mass spectrometry adduct-FIRE-procedure™. To link gene expression to an established phenotypic biomarker of cancer risk, micronuclei frequencies were investigated.

Results: While exposure levels did not differ between sexes at birth, important gender-specific differences were observed in gene expressions associated with these exposures linked with cell cycle, the immune system and more general cellular processes such as (post)-translation. Moreover, oppositely correlating leukemia/lymphoma genes between male and female newborns were identified in relation to the different biomarkers of exposure which might be relevant to male-specific predisposition to develop these cancers in childhood.

Conclusions/Impact: This study reveals different transcriptomic responses to environmental carcinogens between the sexes. In particular, male-specific TNF-alpha-NF-kB signaling upon dioxin exposure and activation of the Wnt-pathway in boys upon acrylamide exposure might represent possible mechanistic explanations for gender specificity in the incidence of childhood leukemia.

1. Introduction

A gender effect in the incidence of childhood cancers has been well described (1, 2). The male-to-female incidence varies for several types of cancers; in particular, a male predominance in the age-adjusted incidence of all types of leukemias and lymphomas is apparent, with the highest ratio (M:F: 3.0) for non-Hodgkin lymphoma (2). Potential gender-specific risk factors include the faster growth rate of the male embryo due to accelerated rates of cell division and proliferation (3). For both sexes, birth weight have been positively associated with childhood leukemia, whereas the maternal dietary intake of DNA topoisomerase II inhibitors was associated with infant leukemia, altogether, they are the most common pediatric neoplasm (4). However, the aetiology of sex-specific childhood cancer still needs to be fully clarified.

In this context, a link between environmental/dietary exposure to carcinogenic chemicals and infant leukemia has been proposed (5). Statistics show significant increases in the incidence of cancer including leukemia, among children in Europe over the last decades (6) suggesting that (maternal) exposure to carcinogenic factors present an aetiological factor. Thus, investigations on possible mechanisms-of-action through which exposure to environmental carcinogens predispose to childhood cancer risk, is warranted. The latent period of e.g. leukemia, its very early onset in childhood might indicate the foetal period to be a critical window of exposure. It has been suggested that such foetal exposure might lead to predisposition to develop disease such as cancer and

immune diseases during childhood or in later life possibly through modulation of the foetal transcriptome (7, 8).

Upon ingestion or inhalation by pregnant women, many environmental carcinogens are capable of crossing the placental barrier and reaching the foetus (9). It has been shown that the placental barrier does not protect the unborn child in case of maternal exposure to acrylamide, dioxins and estrogen-like compounds such as organochlorine pesticides and amphenones (10-13).

Over the last decade, microarray-based genomics technology has significantly progressed enabling whole genome screening at the gene expression level, making this highly suitable for investigating mechanisms underlying e.g. the molecular effects of carcinogenic exposure. Since a gender effect in the incidence of childhood cancers exists, with cancer of the blood system being far more frequent in males than females, we hypothesized that differences at the transcriptomic level in cord blood will occur between male and female newborns as a consequence of foetal carcinogenic exposure, possibly leading to gender-specific predisposition to develop childhood leukemia.

For this, global transcriptomic responses in umbilical cord blood samples from Norwegian male and female newborns were examined in relation to markers of carcinogenic exposure and effect. Investigated non-genotoxic carcinogen exposures determined by means of DR CALUX®, include aryl hydrocarbon (Ah) receptor-mediated compounds such as polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin like polychlorinated biphenyls (PCBs) (14). Measurements by CALUX® assay of ERα

and AR receptor activation represent exposure to endocrine disruptors such as carcinogenic pesticides like 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and (anti-) androgens (15).

Global transcriptomic responses in male and female newborns were also examined in relation to genotoxic acrylamide exposure. Humans are exposed to relatively high doses of acrylamide since this compound is formed in food during cooking (16, 17). Acrylamide can be converted to the epoxide glycidamide by cytochrome P450 2E1 (18). Both compounds are electrophilically reactive. The mutagenicity from acrylamide exposure arises from the reaction of glycidamide with nucleophilic centres in DNA, as the direct interaction of acrylamide with DNA is slow (19). Acrylamide and glycidamide also bind to circulating proteins such as haemoglobin (Hb) and form adducts. This Hb-bound acrylamide represents a well-accepted measure of internal dose and was, as well as the Hb-bound glycidamide, used as a biomarker of exposure to acrylamide in the present study. A recently developed liquid chromatography-tandem mass spectrometry method was used for measurement of the Hb adducts (20).

Gene expression was associated to a validated biomarker of cancer risk i.e. micronuclei frequencies (MN). MN is a well established biomarker of chromosomal breakage and/or whole chromosome loss that are unrepaired, misrepaired or malsegregated due to chromosome, cellular and nuclear dysfunction (21-23), and increased micronucleus frequencies in peripheral blood lymphocytes have been shown in prospective studies to predict the risk of cancer in humans (24).

158 **2. Materials and methods**

159

160 ***2.1 Sample collection***

161 Umbilical cord blood samples were collected immediately after birth from
162 the cord vein of 200 babies whose mothers participated in the Norwegian BraMat
163 cohort. As detailed below in section 2.3, 45 male and 66 female newborns were
164 included in the current study (Table 1). This BraMat sub cohort is nested within
165 the Norwegian Mother and Child cohort (MoBa). Study protocols were approved
166 by the Regional Committee for Ethics in Medical Research in Oslo, Norway.
167 Informed consent was obtained from all participating mothers prior to sample
168 collection. The samples were collected by trained nurses at the maternity wards
169 of the Ulleval and Akershus hospitals in Oslo, Norway. For preserving RNA for
170 microarray analysis, aliquots of heparin-anticoagulated whole blood of 0.4 ml
171 were mixed with 1.2 ml of RNAlater (Ambion/Applied Biosystems, Nieuwerkerk
172 aan den Ijssel, the Netherlands) as soon as possible after blood collection.
173 Samples were kept at -80°C until shipment on dry ice to the research laboratory.
174 The remainder of the whole blood was utilized for CALUX® measurements,
175 assessment of acrylamide- and glycidamide-Hb adducts and analysis of
176 micronuclei frequencies.

177

178 ***2.2 RNA isolation***

179 Total RNA was isolated from the RNAlater-preserved whole blood
180 samples using the RiboPure-Blood system (Ambion) according to the

manufacturer's instructions. RNA yield and purity was measured by means of Nanodrop equipment (Wilmington, DE, USA) and integrity was verified by automated gel electrophoresis (2100 BioAnalyzer, Agilent Technologies, Amstelveen, the Netherlands).

2.3 Gene expression analysis

From the 200 BraMat samples, only RNA samples with a RNA Integrity Number (RIN) ≥6 and a bench time (time from collection until RNA stabilization) of ≤ 6 hours, i.e. 120 samples were used for transcriptomic analysis. Total RNA (1 microgram) was used to generate Cyanine-labeled cRNA using the Agilent Low RNA Input Linear Amplification kit according to the manufacturer's instructions. Each individual cord blood sample was labelled by means of Cyanine-5 and competitively hybridized against a common reference sample (pooled RNA cord blood samples, labelled with Cyanine-3) onto Agilent 4x44k human oligonucleotide microarrays (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions. After hybridization, microarrays were washed and scanned immediately using an Axon GenePix® 4000B Microarray Scanner (Molecular Devices, Sunnyvale, USA). Laser power was set to 100%. The photo multiplier tube (PMT) gain was set to a saturation tolerance of 0.02% to minimize background and saturated spots.

Additional sample exclusions were based on technical performance of the microarrays, as well as on absence of informed consent forms or reported

203 smoking of the mother at 30 weeks of pregnancy. The number of cases left in the
204 study after exclusions was 111.
205
206 ***2.4 Internal measurements of pollutants***
207 Dioxin-like activity in plasma was determined in a total of 43 samples using the
208 dioxin responsive (DR) CALUX® reporter gene bioassay. The bioassay
209 comprises a genetically modified H4IIE rat hepatoma cell line containing the
210 luciferase gene under control of the AhR. Approximately 3 grams of plasma from
211 umbilical cord blood was extracted by means of shake-solvent extraction
212 (hexane:diethylether, 97:3). Extracted fat was used for clean-up on an acid silica
213 column (20% and 33% H_2SO_4), topped with sodium sulphate. Cleaned extracts
214 were dissolved in DMSO (8 µl); the DR CALUX® activity was determined
215 following exposure to dilution series of cleaned extracts (0.8% DMSO) for 24 hrs.
216 Estrogenic and androgenic activity in umbilical cord blood were determined in a
217 total of 34 and 31 samples respectively, using a human U2OS cell line stably
218 transfected with the luciferase gene that is under control of the estrogen receptor
219 alpha (ERα CALUX®) or androgen receptor (AR CALUX®). Approximately 0.5 ml
220 of cord blood plasma was extracted by means of shake-solvent extraction
221 (MTBE; methyltertiairbutylether). Extracts were dissolved in DMSO (40 µl); the
222 ERα- and AR CALUX® activity was determined following 24 hrs of exposure at
223 various dilutions of the re-dissolved extracts (0.1% DMSO). All bioassays were
224 corrected for procedure blanks and performed including a reference compound
225 calibration curve. By interpolation, final results are expressed as reference

compound equivalents (eq.): DR CALUX® are expressed in pg 2,3,7,8-TCDD-like eq. (TEQ)/gram fat, ERα CALUX® in ng 17β-estradiol-like eq. (EEQ)/ml plasma, AR CALUX® in ng dihydrotestosterone-like eq. (AEQ)/ml plasma (25, 26).

2.5 Haemoglobin-adduct levels

Hb-adducts from acrylamide (AA Hb-adducts) and glycidamide (GA Hb-adducts) were measured as internal dose markers of exposure to acrylamide from food. Red blood cells were isolated and stored at -20 C until further processing. Adduct levels to N-terminal valines in Hb were measured in a total of 84 samples applying the "adduct FIRE procedure" using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Shimadzu Prominence, AB SCIEX 3200 qtrap) for final detection (20). Method performance parameters and its application to measurements of acrylamide and glycidamide Hb-adducts in cord blood samples has previously been described (20, 27).

2.6 Cytokinesis-block micronucleus assay

Micronuclei frequencies (MN) per 1000 binucleated cells (‰MNBN) were analyzed in peripheral blood T-lymphocytes of 33 samples. MNBN, in addition to *in vivo* accumulated MN, reflect damage present on DNA or key proteins and expressed as MN during *in vitro* cell division. MN in mononucleated cells (MNMONO) only reflect chromosomal damage before the start of the assay. Since gene expression is not purely a cumulative measure, and MNMONO frequencies were low, MNBN were used. The *in vitro* cytokinesis blocked MN

assay (CBMN assay) was carried out according to the standardized protocol developed for semi-automated image analysis system (Decordier et al. 2009). Sampling and making of cultures occurred in Oslo, Norway. The protocol was performed as recently described by Vande Loock et al (28).

2.7 Data analysis

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (29) and are accessible through GEO Series accession number GSE31836 (30). Scan images of Cy5- and Cy3- channels were loaded into ImaGene software version 8.0.1 (BioDiscovery, El Segundo, USA) for the extraction of raw pixel intensities and local backgrounds. The data were preprocessed using the Refiner Array module from the Genedata Expressionist system (Genedata AG, Basel, Switzerland). Raw data were assessed for quality and preprocessed as follows. Signals were corrected by subtracting backgrounds without producing negative values. For each feature and array, the contrast, i.e., the quality for each signal according to its signal-to-noise ratio, the distortion and imbalance were determined. Defective or saturated features detected by the scanner software or by the Refiner Array module were diagnosed, and masked. LOWESS correction was used to correct all features so that the signal distortion and imbalance of the two channels is minimized. Quality criteria for the data analysis were set as follows: signal-to-noise ratios >2, relative errors <0.5, saturated and features flagged as masked filtered out.

Dose-response relationships were investigated with the available samples by Pearson's correlation analyses of individual gene expressions (Cy5/Cy3 ratios) and individual values for CALUX® measurements, haemoglobin-adducts and micronuclei frequencies, as mentioned in Table 2. Correlation analyses were performed only for transcripts with least 50 % valid expression values

Significantly correlating transcripts were selected using two cut-off's. Minimal correlation coefficients were set to ≥0.60 or ≤-0.60 and an unadjusted p-value of <0.05. To ensure maximum specificity, a higher cut-off of ≥0.75 or ≤-0.75 was used. No false discovery rates were used, since correlation coefficients were considered more important.

To identify biological processes in which significantly correlating genes are involved, the software suite Metacore™ was used. Gene Ontology (GO) terms, pathways and gene networks containing at least two significantly correlating genes and a p-value ≤0.01 were considered significantly enriched. In addition, a false discovery rate cut-off of 0.25 was applied. For enabling further biological interpretation of correlation data, correlation coefficients were uploaded in Tox-Profiler without any statistical pre-selection of correlating genes. Tox-Profiler uses the T-test to score differences between mean input values (usually expression values, but in our case correlation coefficients) of predefined gene sets and that of all other genes (31). For biological interpretation, gene sets based on annotations from different databases were used, i.e. GO terms, KEGG, Biocarta and WikiPathways. To identify significant effects, a Wilcoxon non-parametric statistical test was used, using an unadjusted E-value cut-off of 0.05.

294 To evaluate differences at the transcriptomic level between male and
295 female newborns in relation to blood cancer risk, the effects on human cancer
296 genes, i.e. genes in which mutations that are causally implicated in oncogenesis
297 have been reported, were investigated (32). For this, only genes connected to
298 leukemia and lymphoma were included which showed an opposite correlation
299 coefficient between male and female neonates. Additionally, the correlation
300 coefficient had to be ≥0.60 or ≤-0.60 in either male or female newborns.
301

3. Results

3.1 Biomarker analyses

From a total of 200 samples from the BraMat cohort, 120 RNA samples were selected based on a RNA Integrity Number (RIN) ≥6 and a bench time (time from collection until RNA stabilization) of ≤ 6 hours. These 120 samples were hybridized onto microarrays. Sample/microarray exclusions were based on technical performance of the microarrays, as well as on absence of informed consent forms or reported smoking of the mother at 30 weeks of pregnancy. The number of cases left in the study after exclusions was 111. Characteristics of the remaining study population are presented in Table 1A, for the whole study population as well as for male and female infants separately. All mothers of the included 111 newborns are non-smokers. Table 1B presents RNA Integrity Numbers (RIN) and bench time, i.e. time from collection until RNA stabilization, while Table 1C presents the data distributions for the investigated parameters on CALUX®, Hb-adducts and micronuclei frequencies. The number of available samples for each biomarker varies as presented in Table 1C. For all these measurements, no significant differences were observed between male and female newborns.

3.2 Number of significantly correlating genes

Correlation analyses of gene expression levels with biomarkers of exposure and with MN measured showed variable numbers of significantly correlating genes (Table 2). Due to missing values, the numbers of available samples for correlation analysis vary per biomarker and were significantly lower than the 111 mentioned in Table 1. Overall, separate analyses for male and female newborns resulted in higher numbers of significantly correlating genes per gender with low overlap of similarly expressed genes between the two sexes, thus indicating a clear gender-specific toxicogenomic response.

3.3 Biological evaluation

3.3.1 DR CALUX®

Correlation analysis demonstrated that male infants show a higher number of significantly correlating transcripts in relation to dioxin and dioxin-like exposure, assessed by DR CALUX® assay, when compared to females. For males, functional interpretation revealed significantly affected processes relevant to carcinogenicity, such as programmed cell death/apoptosis, spindle/nuclear division and the ubiquitination–proteasome pathway (Table 3). In females, mainly epigenetic responses were found, such as nucleotide and chromatin assembly (Table 4). The mitotic cell cycle appeared to be affected in both males and females.

Tox-Profiler results showed more processes to be significantly affected in female neonates compared to males (Table 5). Female-specific processes

346 included translation and post-translational modification. Tox-Profiler also
347 confirmed the epigenetic responses in females, although these were also (not-
348 significantly) positively correlated in males. T and B cell receptor Signaling
349 Pathways were found to be suppressed in females. Translational processes were
350 also found for male newborns. Interestingly, these were oppositely regulated
351 when compared to females. Also, proteasome degradation was found to be
352 oppositely regulated between the sexes as well as the TNF-alpha-NF-kB
353 Signaling Pathway.
354

355 ***3.3.2 ERα CALUX®***

356 Female newborns showed a higher number of significantly correlating
357 transcripts in relation to estrogen and estrogen-like exposure when compared to
358 males. Accordingly, more processes were found to be significantly affected in
359 females, of which the majority was immune-related. Other processes included
360 more general (post-) translation events, while estrogen (-like) exposure also
361 appeared to affect apoptosis Table 4). Tox-Profiler-based analysis in particular
362 showed the immune response to be positively correlated in females. Also, more
363 general cellular processes mostly related to transcription and post-translational
364 processes were identified. Interestingly, all processes found using Tox-Profiler
365 were oppositely regulated between the sexes, although mainly reaching
366 significance in females only (Table 5).
367

368 ***3.3.3 AR CALUX®***

Male newborns showed a higher number of significantly correlating genes with AR CALUX® involved in immune response, cell cycle regulation, ubiquitin-proteasomal proteolysis and cytoskeletal-related processes (Table 3). Functional enrichment analyses on significantly correlating genes for females resulted in an extensive list comprising general cellular processes like (post-) translation, metabolic and biosynthesis processes (Table 4). This finding is confirmed by the Tox-Profiler analysis, which also shows general cellular processes to be significantly affected in female newborns (Table 5).

3.3.4 Haemoglobin adduct level

Analyses revealed only weak correlation of GA Hb-adducts with gene expression, leaving too few significantly correlating genes to perform biological interpretation analyses. For AA Hb-adducts, in particular for males, regulation of developmental process, exocytosis and regulation of T-cell activation and neuron differentiation was found (Tables 3 and 4). Tox-Profiler results revealed general processes oppositely regulated between the two sexes, although significance was generally found for females, except for mRNA splicing, which was significantly (negatively) correlated with AA Hb-adducts in males (Table 5).

3.3.5 Micronuclei frequency

Male newborns showed a higher number of genes significantly correlating with micronuclei frequencies, when compared to female newborns (1397 vs 95; Table 2). Functional enrichment analyses using a correlation coefficient cut-off of

0.60 identified general cellular processes like translation and RNA processing, next to a number of immune-related processes. A higher cut-off of 0.75, decreasing the number of genes to 270 and thus increasing specificity, resulted in more specific processes such as nuclear fragmentation during apoptosis and cell cycle regulation (Table 3). Despite the limited number of significantly correlating genes for female newborns, cell cycle regulation and DNA damage removal appeared to be enriched (Table 4). Tox-Profiler analysis confirmed the significant correlation of immune-related processes in males. Also, the translational and post-translational processes found by using significantly correlating transcripts were confirmed, showing negatively correlation with micronuclei frequencies in males, where a significant positive correlation was found in females.

No significant correlations were observed between micronuclei frequencies and the exposure markers when the subjects were grouped together as well as for females separately. Only in males, acrylamide and glycidamide heamoglobin adducts correlated significantly with micronuclei frequencies (CC=0.75 with p=0.019 and CC=0.73 with p=0.025, respectively).

3.3.6 Inversely correlating effects on leukemia- and lymphoma-associated genes

Finally, to evaluate differences at the transcriptomic level between male and female newborns in relation to blood cancer risk, we investigated the effects on human cancer genes. Figure 1 presents the top four (based on deltaCC)

415 oppositely correlating cancer genes between male and female newborns in
416 association with the biomarkers. In total, four transcripts correlated inversely in
417 relation to DR CALUX®, 13 in relation to ERα CALUX®, 7 in relation to AR
418 CALUX®, 18 in relation to micronuclei frequencies, 2 in relation to acrylamide and
419 none in relation to glycidamide (supplementary data Table S4).
420

4. Discussion

Since a gender effect in the incidence of childhood cancers is known to exist, we hypothesized differences at the transcriptomic level in cord blood between male and female newborns as a consequence of foetal carcinogenic exposure. Concordantly, different transcriptomic responses to environmental carcinogens between the sexes were observed. Taking into account that the largest differences between sexes in childhood cancer are found for leukemias and lymphomas (2), and that we measured transcriptomics, exposure biomarkers and effect biomarkers in blood of newborns, this discussion will focus on the relation between transscirptome data and childhood risks for acute lymphoblastic leukemia (ALL).

4.1 DR CALUX®

The non-genotoxic carcinogen exposures determined by means of DR CALUX®, comprise compounds that activate the Ah receptor, such as PCDDs, PCDFs, and dioxin like PCBs (14). In our study, far more genes correlated with DR CALUX® in males than in females (Table 2). In addition, functional interpretation demonstrates large gender differences in dose-dependent transcriptomics responses.

An epigenetic response to Ah-receptor-mediated exposure was found, showing nucleosome-related processes positively correlating with DR CALUX® mainly in females. Effects on the nucleosome are in concordance with the known mechanism-of-action of dioxins; activation of transcription by dioxin is

accompanied by changes in chromatin structure, which depend upon a functional aromatic hydrocarbon (Ah) receptor (33). It is, however, unclear why this is sex-specific and how this could affect ALL susceptibility. The (mitotic) cell cycle was mainly affected in males. Although it is generally accepted that Ah receptor activation causes toxic and carcinogenic effects through its role in detoxification by modulating expression of metabolizing enzymes, activation of the Ah receptor possibly also participates in pathways such as cell cycle regulation, apoptosis and immune response (34). Possibly, the activation of cell cycle regulating genes via the Ah-receptor in male neonates is stronger than in females, and therefore males are more susceptible to develop ALL due to exposure to PCB and other Ah-receptor activators.

T and B cell Receptor Signaling Pathways are negatively correlated with dioxin exposure in female neonates. Foetal TCDD exposure in animals has been linked to inhibition of cellular differentiation and maturation, primarily targeting T lymphocytes, leading to thymic atrophy and immunosuppression in offspring (35). This may suggest that the immune system is more suppressed in females exposed to Ah-activators, than in males. That would imply a higher risk in females, which is not the case in childhood ALL. However, a higher incidence has been reported in female infants (36) possibly suggesting T and B cell Receptor Signaling Pathways to mechanistically underlie the increased risk of developing of infant ALL.

Interestingly, the TNF-alpha-NF-kB Signaling Pathway is negatively correlated with dioxin exposure in females, while (not significantly) positively

correlated in male newborns. The association of inflammation and cancer has been studied extensively (37) showing TNF-*α* acts as a key mediator of inflammation and cancer (38). Although this pathway is involved in general cancer susceptibility, NF-kB activation has been consistently associated with childhood ALL (39, 40). The involvement of the NF-kB pathway has been suggested to play a role in the sex-specific character of childhood leukemia based on the association of the *IRF4* gene with higher male-specific risk with childhood ALL (41). IRF4 was however not found to correlate with dioxin exposure although we found several other genes showing differential gender-specific correlation within the TNF-alpha-NF-kB Signaling Pathway, including e.g. *TRAF1*, *TRAF3, MAP3k3*, *MAP3k14* and *CASP8*. This might further indicate the TNF-alpha-NF-kB Signaling Pathway as a possible contributing factor in relation to the male predominance in childhood ALL. Moreover, the unaffected or even non-significantly increased signaling by tumor necrosis factor (TNF)-α in males might also result in a failure of the lymphocytes to undergo apoptosis (42), possibly contributing to the increased risk of childhood ALL in males. This is underlined by the male-specific negative correlation of *CASP8* with dioxin exposure. Accordingly, apoptosis was found to be exclusively affected in males. Nine human blood cancer-associated genes were found to be inversely correlating strongly indicating a gender-specific response to dioxins in relation to tumorigenesis. The human anti-apoptotic cancer genes *JAK2* and *VHL* are positively correlated with dioxin exposure in males, while negatively in females contributing to apoptosis blockade, possibly predisposing males to develop

childhood ALL. These cancer genes have been associated with childhood ALL (43-44).

In conclusion, the female-specic suppression of the TNF-alpha-NF-kB Signaling Pathway upon dioxin exposure might protect female neonates from developing childhood ALL, while their male counterparts are at higher risk, possibly due to consequent blockade of apoptosis.

4.2 ERα CALUX®

Estrogens have been linked to B and T cell activation, enhanced antigen presentation by dendritic cells, and cytokine production (45). Accordingly, we found immune-related processes to be affected, with a higher response in females compared to males. In fact, results using unfiltered correlation data suggest an inverse regulation upon estrogen receptor activation with a significant stimulation of the GO term 'immune system' in females and (non-significant) suppression in males. GO terms like B and T cell signalling show similar inverse trends. The male-specific immune suppression may contribute to the higher incidence of childhood ALL since leukemia is associated with immune dysfunction (47). Further confirming gender-specificity, we found leukemia- and lymphoma-related genes differentially correlating with estrogen exposure (Table 7). Not all genes conclusively point to a male predisposition for developing ALL, in fact we found a female-specific positive correlation of *MllT6.* Mixed lineage leukemias are histone methyl-transferases that regulate gene activation and are also well known to be rearranged in acute myeloid and lymphoid leukemias (47).

Interestingly, the female-specific correlation might be in line with the literature: Unlike childhood leukemia diagnosed in childhood, where there is a predominance in males, high frequencies of MLL rearrangements in female Infant Leukemia have been reported, as well as a higher risk for females to develop infant ALL and (36, 48).

4.3 AR CALUX®

Next to androgens, certain environmental pollutants may influence AR signaling pathways by acting as or interfering with endogenous androgens, regulating the expression of various genes involved in cell cycle control, apoptosis, cell growth and differentiation (49). We found the cell cycle GO term to correlate significantly to AR CALUX® in males. Possibly, the activation of cell cycle genes via the AR-receptor in male neonates is stronger than in females, and leading to a higher males-specific susceptibility. In contrast, the immune response was significantly suppressed in only females implying a higher cancer risk in females. Concordantly, differentially correlating leukemia and lymphoma genes with AR receptor activation do not conclusively support a male predisposition. In fact, next to the *MLLT6* gene correlating with the ERα receptor, a female-specific positive correlation of *MllT3* with AR receptor activation was found which might be related to female predisposition to develop infant leukemia (36, 47, 48).

4.4 Hb-adducts

Tox-Profiler revealed the Wnt signaling pathway and a number of general processes oppositely regulated between the two sexes upon acrylamide exposure. Aberrations of the Wnt pathway have been associated with various types of cancer including ALL. A male-specific activation of the Wnt pathway might thus contribute to the higher incidence of cancer in the blood system.

4.5 Micronuclei frequencies

Micronuclei formation in peripheral blood T-lymphocytes may be regarded as a phenotypic endpoint that reflects the deleterious effects on chromosomes due to the (combined) exposure to genotoxic carcinogens (50). Previous studies report that the baseline MN frequency is relatively low in newborns and no differences in gender have been identified (28, 51), which is confirmed in our study. Despite this, the sex-specific transcriptomic profiles associated with micronuclei frequencies differ dramatically. Overall, a higher number of significantly correlating genes in male newborns in relation to MN was observed. Correlations were frequently opposite between males and females. The male-specific profile contains many more genes relevant to ALL and carcinogenicity in general. This might contribute to the higher childhood cancer risk in males. Interestingly, 6 genes of our MN correlating genes are also present in the recently published MN gene network (52). They were found to be oppositely correlating between male and female neonates, i.e. *BCL2*, *CDC20*, *PTTG1, BAX*, *PCNA*, and *LMNA*, suggesting sex-specific contribution to the network. Lastly, MN-associated sex-specific differences in general cellular processes such as

559 transcription, post-translational processes, RNA processing and splicing might be
560 of relevance with regard to the reported gender differences in the incidence of
561 childhood cancers, however interpreting these findings is difficult.
562
563 ***45. Conclusions***
564 While exposure to acrylamide and endocrine disruptors did not differ
565 significantly between sexes at birth, distinct gender-specific dose-response
566 related transcriptomic profiles were identified. In particular, male-specific TNF-
567 alpha-NF-kB signaling upon dioxin exposure and subsequent failure of the
568 lymphocytes to undergo apoptosis, and activation of the Wnt pathway in boys
569 upon acrylamide exposure might represent possible mechanistic explanations for
570 the gender-specificity in the incidence of childhood ALL. Interestingly, oppositely
571 correlating leukemia and lymphoma genes between male and female newborns
572 were identified in relation to the different biomarkers of exposure which might be
573 relevant to male-specific predisposition to develop childhood ALL and female-
574 specific predisposition to develop infant ALL .
575 Our study has the strength of considering multiple parameters in relation
576 to gene expression, although we recognize that the correlation analyses in the
577 current study of gene expression with the various biomarkers are based on a
578 relatively small number of samples making multivariate correlation analyses
579 impractical. This is due to the limited availability of samples with biomarker
580 measurements and gender-specific analyses, which reduces statistical power.
581 Nevertheless, since the gender-specific responses occur for multiple biomarkers,

582 we are confident about our conclusions. To confirm the generated hypotheses,
583 future research based on higher number of samples is needed.
584

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752

753 **Tables**

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755 bench time (B) and data distribution of the various parameters (C).

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759

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766 Table 5. GO terms and pathways enriched for males among all correlating genes
767 using Tox-Profiler.

768

769

770 Figure 1. Human leukemia and lymphoma associated genes with opposite
771 correlations with biomarkers between male and female newborns. Only the top 4,
772 based on deltaCC is presented; for complete lists refer to supplementary data
773 Table S4. * indicates a significant correlation, with an unadjusted p-value < 0.05.
774 ** indicates the query terms used to search the GO term annotations for each
775 gene.

Table 1. Characteristics of the study population (A), RNA Integrity Number and bench time (B) and data distribution of the various parameters (C).

A

	All	**Males**	**Females**
N	111	45	66
Birth weight	3629 ± 434	3722 ± 416	3564 ± 438
Gestation	39.8 ± 1.1	39.9 ± 1.1	39.7 ± 1.1
Age - mother	31.3 ± 4.2	31.0 ± 4.3	31.5 ± 4.2
- father	32.8 ± 6.4	32.3 ± 4.3	33.7 ± 5.7
Mother BMI BP	23.4 ± 3.9	23.1 ± 3.6	23.6 ± 4.1
Mother was pregnant before			
- no	35%	34%	36%
- yes	65%	66%	64%
Mother smoked BP			
- no	84%	82%	85%
- occasional	9%	11%	8%
- daily	7%	7%	6%
Delivery start			
- Spontaneous	81%	86%	77%
- Induced	12%	7%	16%
- Caesarean section	7%	7%	8%
Caesarean sections			
- Planned	7	2	5
- Emergency	7	4	3

B

RIN (0-10)	8.0 ± 1.3	7.98 ± 1.30	7.97 ± 1.30
Bench time	77.8 ± 172.4	58.77 ± 116.66	90.63 ± 201.29

C

DR CALUX®	36.4± 9.8 (n=43)	37.4 ± 10.5 (n=14)	35.9 ± 9.6 (n=29)
ERα CALUX®	15.6 ± 14.4 (n=34)	16.1 ± 10.6 (n=17)	15.2 ± 17.8 (n=17)

AR CALUX®	0.08 ± 0.06 (n=31)	0.07 ± 0.03 (n=16)	0.09 ± 0.08 (n=15)
AA Hb-adducts	16.5 ± 6.6 (n=84)	16.0 ± 7.1 (n=34)	16.7 ± 6.4 (n=50)
GA Hb-adducts	10.0 ± 4.0 (n=84)	9.5 ± 3.8 (n=34)	10.3 ± 4.0 (n=50)
‰MNBN	1.2 ± 0.9 (n=33)	1.1 ± 0.8 (n=13)	1.2 ± 0.9 (n=16)

Data are presented as mean ± SD unless stated otherwise. BP: before pregnancy, RIN: RNA Intergrity Number, Bench time: time from blood collection to RNA stabilisation. Birth weight expressed in g, gestation in weeks, age in years, bench time in minutes, DR CALUX® in pg TEQ/gr fat, ERα CALUX® (ng EEQ/ml plasma), AR CALUX® (ng AEQ/ml plasma), Hb-adducts in pmol/g Hb, ‰MNBN in MN per 1000 binucleated cells.

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	# of genes	Pos. correlation	Neg. correlation	Gender	# of genes	Pos. correlation	Neg. correlation	Overlap boys-girls
DR CALUX®	**3**	0	3	Boys (n=14)	**371**	163	208	1
				Girls (n=29)	**39**	29	10	
ERα CALUX®	**20**	14	6	Boys (n=17)	**493**	311	182	3
				Girls (n=17)	**626**	459	167	
AR CALUX®	**83**	69	14	Boys (n=16)	**1293**	946	347	29
				Girls (n=15)	**508**	366	142	
AA Hb-adducts	**0**	0	0	Boys (n=34)	**23**	18	5	0
				Girls (n=50)	**0**	0	0	
GA Hb-adducts	**0**	0	0	Boys (n=34)	**6**	2	4	0
				Girls (n=50)	**0**	0	0	
‰MNBN	**22**	4	18	Boys (n=13)	**1397**	662	735	1
				Girls (n=16)	**95**	28	67	

Numbers are presented for sexes grouped together and separately, correlation coefficients >0.60 or <-0.60 and an unadjusted p-value ≤0.05).

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Table 3. The GO terms, pathways and networks enriched among the significantly correlating genes for the various biomarkers for males

GO term / Pathway / Network	# significant processes (CC≥0.60/ CC≥0.75)	P-value (# genes affected/total # in pathway)	Database
DR CALUX®	29/16		
cell cycle		3.76E-04 (31/679)	GO
apoptosis		5.63E-04 (26/543)	GO
ERα CALUX®	50/9		
antigen processing and presentation		2.92E-05 (9/61)	GO
regulation of adaptive immune response		1.69E-04 (8/60)	GO
AR CALUX®	7/1		
Cell cycle_G1-S		6.64E-03 (17/122)	Pathways
Proteolysis_Ubiquitin-proteasomal proteolysis		7.11E-03 (19/143)	Pathways
AA Hb-adducts	4/0		
regulation of developmental process		5.51E-03 (4/637)	GO
regulation of T cell activation		8.89E-03 (2/121)	GO
GA Hb-adducts	0/0		
‰MNBN	22/53		
DNA damage_Checkpoint		9.22E-03 (16/92)	Networks
regulation of cell cycle[a]		3.49E-04 (14/314)	GO
Apoptosis and survival_p53-dependent apoptosis[a]		1.06E-03 (4/25)	Pathways

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S1.
[a]Found when using the 0.75 correlation coefficient cut-off.

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Table 4. The GO terms, pathways and networks enriched for males among the significantly correlating genes for the various biomarkers for females

GO term / Pathway / Network	# significant processes (CC≥0.60/ CC≥0.75)	P-value (# genes affected/total # in pathway)	Database
DR CALUX®	18/0		
nucleosome assembly		1.22E-05 (4/69)	GO
Cell cycle_Initiation of mitosis		6.37E-03 (2/18)	Pathways
ERα CALUX®	74/88		
immune system process		8.49E-06 (58/863)	GO
regulation of lymphocyte activation		1.99E-05 (18/155)	GO
AR CALUX®	39/33		
translational elongation		8.72E-19 (28/104)	GO
ubiquitin-dependent protein catabolic process		4.70E-06 (21/214)	GO
AA Hb-adducts	0/0		
GA Hb-adducts	0/0		
‰MNBN	3/6		
nucleotide-excision repair, DNA damage removal		1.30E-04 (3/18)	GO
Cell cycle_Initiation of mitosis		6.37E-03 (2/18)	Pathways

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S2.

Table 5. GO terms and pathways enriched for males among all correlating genes using Tox-Profiler.

Biomarker	Process	# significant processes males/females	T-value males	p-value males	T-value females	p-value females	Database
DR CALUX®		5/29					
	nucleosome assembly		4.1	0.154	6.4	<0.001	GO
	T cell receptor signaling pathway		-2.7	1.000	-4.5	0.001	KEGG
	B cell receptor signaling pathway		-0.6	1.000	-4.1	0.005	KEGG
	TNF-alpha-NF-kB Signaling Pathway		2.9	0.551	-4.2	0.010	WikiPathways
ERα CALUX®		2/41					
	immune response		-1.4	1.000	6.8	<0.001	GO
	T Cell Receptor Signaling Pathway		-1.5	1.000	6.8	<0.001	WikiPathways
	B Cell Receptor Signaling Pathway		-1.9	1.000	4.9	<0.001	WikiPathways
AR CALUX®		12/24					
	immune response		-2.5	1.000	-6.6	<0.001	GO
	ubiquitin-dependent protein catabolic process		0.9	1.000	5.9	<0.001	GO
	nucleosome		-4.7	0.020	-1.2	1.000	GO
AA Hb-adducts		4/17					
	translational elongation		-1.7	1.000	8.8	<0.001	GO
	RNA splicing		-5.6	<0.001	1.7	1.0000	GO
GA Hb-adducts		8/12					
	Wnt signaling pathway		4.2	0.032	0.2	1.000	KEGG

‰MNBN		30/13					
	translational elongation		8.5	<0.001	-5.7	<0.001	GO
	Spliceosome		4.6	0.002	-5.4	<0.001	KEGG
	mRNA processing		4.0	0.012	-4.1	0.002	WikiPathways
	Pathways in cancer		4.8	0.001	1.8	1.000	KEGG
	translational elongation		8.5	<0.001	-5.7	<0.001	GO

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S3.

Tables

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Table 5. GO terms and pathways enriched for males among all correlating genes using Tox-Profiler.

Table 6. Human leukemia and lymphoma associated genes with opposite correlations with biomarkers between male and female newborns

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Mother was pregnant before			
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- yes	65%	66%	64%
Mother smoked BP			
- no	84%	82%	85%
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Delivery start			
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B

RIN (0-10)	8.0 ± 1.3	7.98 ± 1.30	7.97 ± 1.30
Bench time	77.8 ± 172.4	58.77 ± 116.66	90.63 ± 201.29

C

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ERα CALUX®	15.6 ± 14.4 (n=34)	16.1 ± 10.6 (n=17)	15.2 ± 17.8 (n=17)

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				Girls (n=50)	**0**	0	0	
GA Hb-adducts	**0**	0	0	Boys (n=34)	**6**	2	4	0
				Girls (n=50)	**0**	0	0	
‰MNBN	**22**	4	18	Boys (n=13)	**1397**	662	735	1
				Girls (n=16)	**95**	28	67	

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Table 3. The GO terms, pathways and networks enriched among the significantly correlating genes for the various biomarkers for males

GO term / Pathway / Network	# significant processes (CC≥0.60/ CC≥0.75)	P-value (# genes affected/total # in pathway)	Database
DR CALUX®	29/16		
cell cycle		3.76E-04 (31/679)	GO
apoptosis		5.63E-04 (26/543)	GO
ERα CALUX®	50/9		
antigen processing and presentation		2.92E-05 (9/61)	GO
regulation of adaptive immune response		1.69E-04 (8/60)	GO
AR CALUX®	7/1		
Cell cycle_G1-S		6.64E-03 (17/122)	Pathways
Proteolysis_Ubiquitin-proteasomal proteolysis		7.11E-03 (19/143)	Pathways
AA Hb-adducts	4/0		
regulation of developmental process		5.51E-03 (4/637)	GO
regulation of T cell activation		8.89E-03 (2/121)	GO
GA Hb-adducts	0/0		
‰MNBN	22/53		
DNA damage_Checkpoint		9.22E-03 (16/92)	Networks
regulation of cell cycle[a]		3.49E-04 (14/314)	GO
Apoptosis and survival_p53-dependent apoptosis[a]		1.06E-03 (4/25)	Pathways

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S1.
[a]Found when using the 0.75 correlation coefficient cut-off.

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Table 4. The GO terms, pathways and networks enriched for males among the significantly correlating genes for the various biomarkers for females

GO term / Pathway / Network	# significant processes (CC≥0.60/ CC≥0.75)	P-value (# genes affected/total # in pathway)	Database
DR CALUX®	18/0		
nucleosome assembly		1.22E-05 (4/69)	GO
Cell cycle_Initiation of mitosis		6.37E-03 (2/18)	Pathways
ERα CALUX®	74/88		
immune system process		8.49E-06 (58/863)	GO
regulation of lymphocyte activation		1.99E-05 (18/155)	GO
AR CALUX®	39/33		
translational elongation		8.72E-19 (28/104)	GO
ubiquitin-dependent protein catabolic process		4.70E-06 (21/214)	GO
AA Hb-adducts	0/0		
GA Hb-adducts	0/0		
‰MNBN	3/6		
nucleotide-excision repair, DNA damage removal		1.30E-04 (3/18)	GO
Cell cycle_Initiation of mitosis		6.37E-03 (2/18)	Pathways

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S2.

Table 5. GO terms and pathways enriched for males among all correlating genes using Tox-Profiler.

Biomarker	Process	# significant processes males/females	T-value males	p-value males	T-value females	p-value females	Database
DR CALUX®		5/29					
	nucleosome assembly		4.1	0.154	6.4	<0.001	GO
	T cell receptor signaling pathway		-2.7	1.000	-4.5	0.001	KEGG
	B cell receptor signaling pathway		-0.6	1.000	-4.1	0.005	KEGG
	TNF-alpha-NF-kB Signaling Pathway		2.9	0.551	-4.2	0.010	WikiPathways
ERα CALUX®		2/41					
	immune response		-1.4	1.000	6.8	<0.001	GO
	T Cell Receptor Signaling Pathway		-1.5	1.000	6.8	<0.001	WikiPathways
	B Cell Receptor Signaling Pathway		-1.9	1.000	4.9	<0.001	WikiPathways
AR CALUX®		12/24					
	immune response		-2.5	1.000	-6.6	<0.001	GO
	ubiquitin-dependent protein catabolic process		0.9	1.000	5.9	<0.001	GO
	nucleosome		-4.7	0.020	-1.2	1.000	GO
AA Hb-adducts		4/17					
	translational elongation		-1.7	1.000	8.8	<0.001	GO
	RNA splicing		-5.6	<0.001	1.7	1.0000	GO
GA Hb-adducts		8/12					
	Wnt signaling pathway		4.2	0.032	0.2	1.000	KEGG

‰MNBN		30/13					
	translational elongation		8.5	<0.001	-5.7	<0.001	GO
	Spliceosome		4.6	0.002	-5.4	<0.001	KEGG
	mRNA processing		4.0	0.012	-4.1	0.002	WikiPathways
	Pathways in cancer		4.8	0.001	1.8	1.000	KEGG
	translational elongation		8.5	<0.001	-5.7	<0.001	GO

Only processes mentioned in the text are presented; for complete lists refer to supplementary data Table S3.

Fig 1

	GeneSymbol	Agilent Gene ID	CC Males	CC Females	Regulation of progression through cell cycle**	Proliferation/replication**	Apoptosis**	Regulation of transcription**	Cromatin/histone **	Immune response **
DR										
	JAK2	Janus kinase 2 a protein tyrosine kinase	0.77*	-0.18						
	EWSR1	Ewing sarcoma breakpoint region 1	0.69*	-0.20						
	HSP90AB1	heat shock protein 90kDa alpha cytosolic, class B member 1	0.62*	-0.26						
	ZNF384	zinc finger protein 384	0.71*	-0.12						
Era										
	FUS	fusion involved in t 12;16 in maligt liposarcoma	-0.66*	0.70*						
	CD79B	CD79b molecule, immunoglobulin-associated beta	-0.50	0.70*						
	LCK	lymphocyte-specific protein tyrosine kinase	-0.25	0.80*						
	MLLT6	myeloid/lymphoid or mixed-lineage leukemia trithorax homolog, Drosophila; translocated to, 6	0.39	-0.63*						
AR										
	DEK	DEK oncogene DNA binding	-0.43	0.72*						
	MLLT3	myeloid/lymphoid or mixed-lineage leukemia trithorax homolog, Drosophila; translocated to, 3	-0.50	0.65*						
	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	-0.49	0.63*						
	IGH@	immunoglobulin heavy locus	0.62*	-0.41						
‰MNBN										
	BCL2	B-cell CLL/lymphoma 2	-0.77*	0.49						
	RUNX1	runt-related transcription factor 1 acute myeloid leukemia 1; aml1 oncogene	-0.80*	0.33						
	TPM3	tropomyosin 3	0.72*	-0.34						
	EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	0.69*	-0.35						
AA Hb-adducts										
	NIN	ninein GSK3B interacting protein	-0.64*	0.12						
	PBX1	pre-B-cell leukemia homeobox 1	0.66*	-0.09						
GA Hb-adducts										

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Global gene expression analysis in cord blood reveals gender-specific differences in response to carcinogenic exposure *in utero*

Kevin Hochstenbach, Danitsja M. van Leeuwen, Hans Gmuender, et al.

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