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# Genetic Reprogramming of Human Mammary Cells by Environmental Carcinogens Released into Breast Milk

Ramiro Dip[a], Tonko Buterin[a], Daniela Wenger[b], Peter Schmid[b], and Hanspeter Naegeli*[a]

*Abstract:* Epidemiologic studies indicate that high serum levels of chlorinated pollutants such as dioxins or pesticides constitute a risk factor for breast cancer in postmenopausal women. This finding has been attributed to the ability of organochlorine compounds to induce the expression of cytochrome P450 (CYP) enzymes, which are thought to increase the burden of genotoxic metabolites. However, it was not clear whether the incriminated pollutants accumulate to sufficiently high levels within the human body to be able to promote significant transcriptional responses. Here, milk samples from nursing mothers were processed by gel permeation chromatography to isolate complex mixtures of residues containing both endogenous hormones and lipophilic contaminants of environmental origin. High-density oligonucleotide microarrays were used to monitor global transcriptional profiles in a human cell line (MCF7) that represents one of the most frequently used model systems to study breast cancer biology. We found that all breast milk extracts were able to reprogram the genome of MCF7 cells by imposing a typically estrogenic expression fingerprint. This estrogenic background was overlapped only by the induction of transcripts coding for *CYP1A1* and, to a minor degree, *CYP1B1*. The magnitude of induction of these metabolic enzymes correlated with the respective organochlorine concentrations. Thus, in support of previous epidemiologic studies, we demonstrate that contaminants released from human adipose tissue trigger a potentially genotoxic pathway in cells from the mammary gland.

**Keywords:** Dioxin · Endocrine disruptors · Estrogen · PCB · TCDD

## 1. Introduction

Breast cancer is the most frequent neoplastic disease in women worldwide. Some factors modulating the breast cancer risk have been identified, including obesity, genetic predisposition, age at menarche and menopause, breastfeeding or postmenopausal hormone replacements. However, the etiology of breast cancer remains largely elusive as these known risk factors explain only a fraction of all cases.[1–3] There is also concern regarding the risk associated with chlorinated organic pollutants commonly found in the human body. Examples of such anthropogenic compounds are the pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT), its major metabolite 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene (DDE), industrial chemicals such as polychlorinated biphenyls (PCBs), as well as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) arising from waste incineration.[4,5] These chlorinated chemicals persist in the environment, magnify along the food chain and, owing to their lipophilicity, undergo lifelong storage in adipose tissues.[6] Because the use of DDT and PCBs has been banned in many countries since the 1970s, their concentration in human tissues is slowly declining.[7] An opposite trend of increasing concentrations is observed for polybrominated diphenyl ethers (PBDEs).[8,9]

The contribution of organochlorine pollutants to the pathogenesis of breast cancer has been investigated in several studies, (reviewed by Tsuda *et al.*[10]). No association has been found between PCDD/PCDF serum levels and breast cancer.[11,12] Similarly, in most reports no correlation could be detected between breast cancer and the levels of DDT or DDE.[13–16] In contrast, there is a breast cancer predisposition in postmenopausal women carrying high serum levels of PCBs together with particular genetic polymorphisms in *CYP1A1*, a member of the cytochrome P450 (CYP) family.[1,3,17] As a molecular basis to explain these epidemiologic findings, it has been proposed that the up-regulation of *CYP1A1*, which is inducible by PCBs and other organochlorine compounds,[18,19] may lead to the excessive formation of reactive metabolites that cause DNA damage and genetic mutations. This model predicts that populations with higher *CYP1A1* or *CYP1B1* activity would be at increased risk of breast cancer.[20]

Two important questions remained unanswered. First, it has always been inferred but never demonstrated that organochlorine compounds reach sufficiently high levels in the mammary gland to trigger adverse metabolic effects. Second, it is well known that humans are usually exposed to a combination of substances, such that multiple contaminants and metabolites may give rise

*Correspondence: Prof. Dr. H. Naegeli[a]
Tel.: +41 44 635 8763
Fax: +41 44 635 8910
E-mail: naegelih@vetpharm.uzh.ch
[a]Institute of Pharmacology and Toxicology
University of Zürich-Vetsuisse
Winterthurerstrasse 260
CH-8057 Zürich
[b]Laboratory of Analytical Chemistry
Swiss Federal Institute for Materials Science and Technology
Überlandstrasse 129
CH-8600 Dübendorf

to antagonistic interactions that ultimately mitigate their potential toxicity. For example, estrogens have been shown to suppress *CYP1A1* expression,[20–22] suggesting that the endogenous hormone 17β-estradiol or estrogen-like contaminants counteract a critical biological endpoint of dioxins, PCBs and other CYP inducers. To address these questions, we sought to examine the transcriptional changes elicited by complex mixtures of pollutants that accumulate in the human body. Although persistent organochlorines display long half-lives,[23] considerable amounts are transferred across the mammary gland,[6,8] such that the lipid fraction of breast milk contains contaminants at concentrations similar to those in other fatty compartments. As a consequence, breast milk constitutes a convenient and non-invasive matrix to assess the internal burden of lipophilic chemicals.

The transcriptional effects of breast milk have never been tested on cells from the mammary gland, but conflicting results have been observed in standard bioassays. Some studies showed that breast milk induces the expression of *CYP1A1* or analogous reporter genes, primarily the dioxin-responsive chemically activated luciferase (DR-CALUX) construct, in rat hepatoma cells.[24–26] However, a more recent contradictory study showed that breast milk, unlike formula, is unable to induce *CYP1A1* expression in human hepatoma cells.[27] In view of these conflicting findings, we used a representative human cell line derived from the mammary epithelium to assess global transcriptional changes induced by lipophilic breast milk extracts.

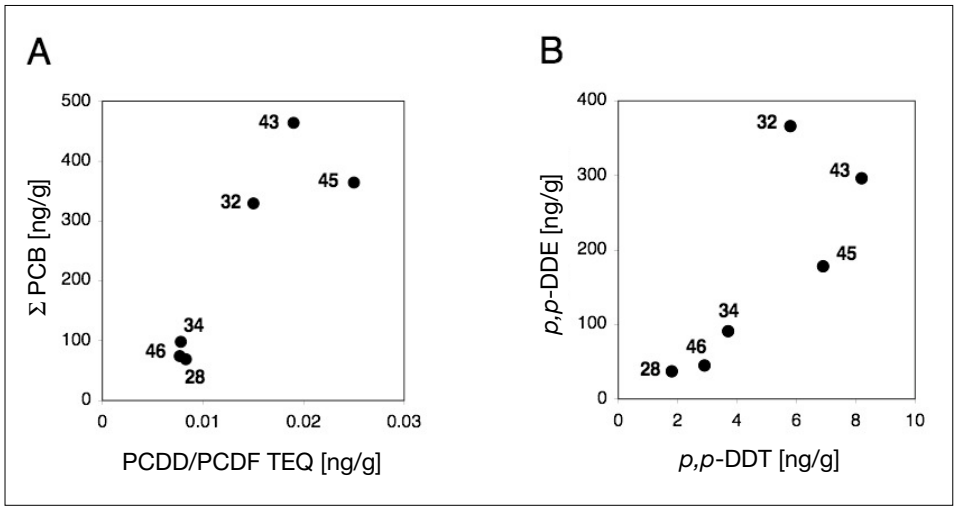


Fig. 1. Major chlorinated contaminants in the lipid fraction of breast milk. (A) Concentration of PCBs (expressed as the sum of congeners 28, 52, 101, 118, 138, 153 and 180) compared to the concentration of PCDDs and PCDFs (expressed as WHO-TEQ values). (B) Concentration of DDT and its major metabolite DDE. These graphical representations illustrate the difference between the less contaminated reference samples (no. 28, 34 and 46) and the more contaminated samples no. 32, 43 and 45. PBDEs were detected at considerably lower concentrations than chlorinated chemicals, *i.e.* between 2.2 ng/g lipid in sample no. 28 and 10.0 ng/g in sample no. 34.

## 2. Results

### 2.1. *Fractionation and Chemical Analysis*

The concentration of environmental pollutants was determined in the lipid phase of 36 milk samples collected from breastfeeding women in 2002. The samples were fractionated by gel permeation chromatography to obtain residue mixtures comprising a wide spectrum of organic contaminants. The analytes detected by gas chromatography/high resolution mass spectrometry include a range of lower and higher chlorinated PCB congeners (from PCB-28 to PCB-180), 2,3,7,8-substituted PCDDs and PCDFs, DDT, its metabolite DDE, and the most prevalent PBDE congeners (from BDE-28 to PBDE-209). These measurements yielded a group of three samples with very low contaminant levels (no. 28, 34 and 46), representing the reference group, and a second group consisting of the most heavily contaminated samples (no. 32, 43 and 45). Compared to the reference group, the contaminated milk extracts displayed more than three-fold increased levels of PCBs, PCDDs and PCDFs (Fig. 1A) and at least 4-fold higher levels of DDT and DDE (Fig. 1B). These two sample groups containing low and high pollutant levels were used for the subsequent gene expression studies.

### 2.2. *Genome-wide Expression Changes*

MCF7 cells were incubated directly with the residue mixtures separated from breast milk lipids by gel permeation chromatography. In triplicate experiments, the cell culture medium was supplemented with lipophilic extracts to achieve a contamination level that is equivalent to that found in breast milk with a 5% fat content. After 24 h exposures in reconstituted medium, the cells were harvested and processed for the genome-wide determination of expression profiles using DNA microarrays that cover the sequences of 47'000 human transcripts. The hybridization results were normalized and subsequently filtered using, as the statistical cut-off, a significance value of $P < 0.05$ (ANOVA) in at least one of the treatment groups. Initially, the data output was simplified by focusing on transcripts that, upon treatment with at least one of the extracts, show a minimal fold change > 5 relative to the solvent controls. This analysis yielded 57 distinct transcripts that were substantially regulated upon incubation with breast milk extracts (Fig. 2).

The observed expression changes were almost exclusively characterized by the induction of genes such as *RRM2* (ribonucleotide reductase M2), *TYMS* (thymidylate synthetase), *TK1* (thymidine kinase 1), *CCNA2* (cyclin A2) or *MCM10* (minichromosome maintenance 10) that take part in a well known mitogenic response to estrogenic stimuli.[28,29] The most pronounced up-regulation of estrogen-dependent factors was observed after incubation with extract no. 46, belonging to the reference group of poorly contaminated samples. A side-by-side comparison illustrates that the transcriptional profile induced by this particular extract is nearly identical to that elicited by the endogenous 17β-estradiol at the near saturating concentration of 30 pM (Fig. 2). This estrogenic expression pattern was also found after incubation with all other extracts, although with lower amplitudes of regulation.

Another comparison of the same 57 transcripts, in the form of a scatter plot, confirmed that extract no. 46 induces essentially identical transcriptional effects as 17β-estradiol (Fig. 3A). However, no direct relationship could be found between the induction of these estrogen-dependent transcripts and the presence of major environmental contaminants. This lack of a positive correlation is evidenced by a comparison between the RRM2 transcripts and the concentration of PCBs (Fig. 3B), PCDDs/PCDFs (Fig. 3C) or DDT/DDE (Fig. 3D). The distinctive estrogenic background, on the other hand, prompted us to pool the breast milk samples in order to measure their 17β-estradiol content by liquid chromatography/tandem mass spectrometry. This analysis demonstrated that the samples contained sufficient 17β-estradiol to yield a mean concentration in the culture medium of 6 pM or higher (see Discussion).

The only exception to the overall estrogenic fingerprint induced by breast milk extracts consisted of an up-regulation of

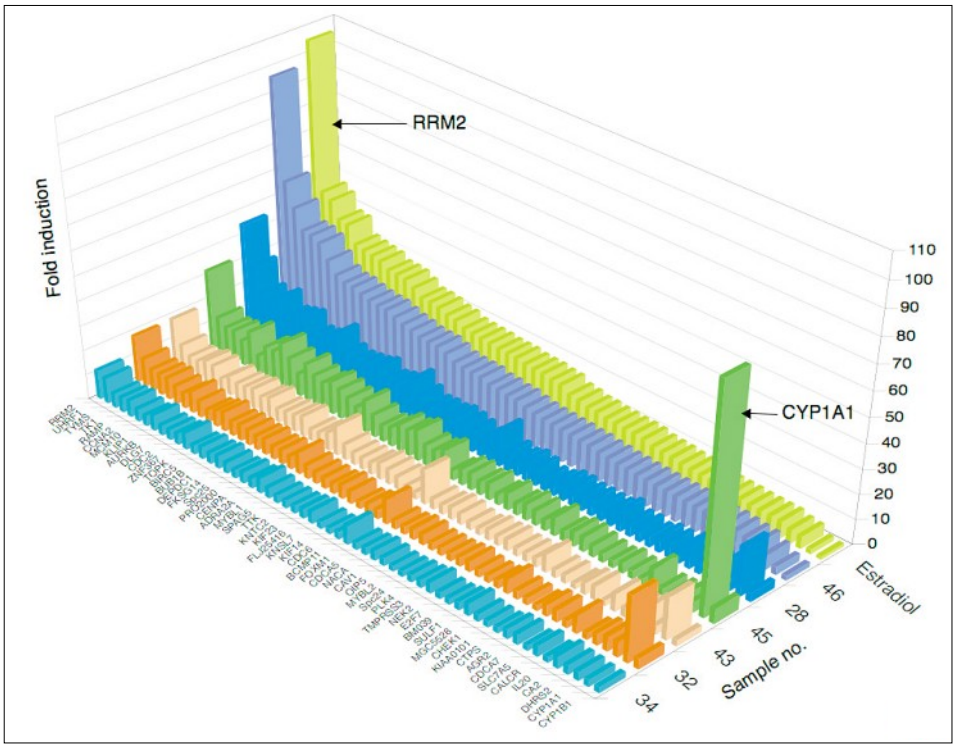


Fig. 2. Transcriptional changes (mean values of three independent experiments) resulting from the incubation of human MCF7 cells with breast milk extracts or a 17β-estradiol standard (30 pM). The fold changes have been determined relative to solvent controls incubated with 0.3% (v/v) DMSO. The microarray hybridization data were analyzed using the GeneSpring software. Only transcripts with a fold change > 5 (statistical cut-off: $P$ <0.05) in at least one of the treatment groups are shown. The transcripts were arranged following their descending order in the experiments with sample no. 46.

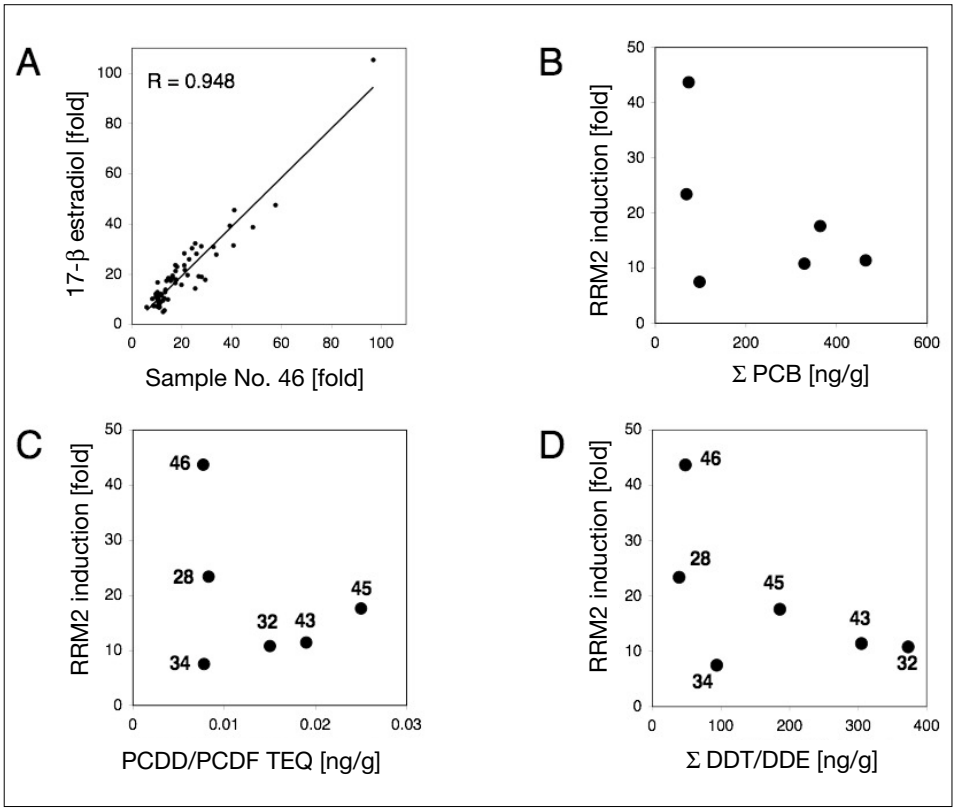


Fig. 3. Estrogenic response induced in MCF7 cells by breast milk extracts. (A) Scatter plot comparing 57 estrogen-regulated transcripts (fold change > 5 in one of the treatment groups; $P$ <0.05). X-axis, transcriptional induction following treatment with sample no. 46; Y-axis, induction of the same transcripts after incubation with 17β-estradiol (30 pM). R, correlation coefficient. (B) Degree of *RRM2* induction in relation to the PCB concentrations (sum of congeners 28, 52, 101, 118, 138, 153 and 180). (C) *RRM2* induction in relation to PCDD/PCDF levels (expressed as TEQ concentrations). (D) *RRM2* induction plotted against the concentration of DDT and DDE.

transcripts coding for two members of the CYP family, *i.e. CYP1A1* and *CYP1B1*. The strongest induction of these metabolic enzymes was detected following treatment with the highly contaminated extract no. 45 (Fig. 2). This induction of *CYP1A1* and *CYP1B1*, observed in the presence of breast milk residues, was not decreased by co-incubation with the estrogen antagonist ICI 182,780. In contrast, all other transcripts regulated by treatment with milk extracts no. 32, 43 and 45, belonging to the highly contaminated group, were diminished or suppressed by the addition of ICI 182,780 (Fig. 4). These results confirm that the induction of *CYP1A1* and *CYP1B1* is independent of signaling pathways driven by estrogen receptors.

Subsequently, the microarray data were reinvestigated to include transcripts with less than five-fold changes relative to the solvent controls. This systematic analysis revealed that no messengers, other than those coding for CYP enzymes and a large number of estrogen-dependent factors, were significantly ($P$ <0.05) up- or down-regulated following the treatment with breast milk extracts (data not shown).

### 2.3. *Validation of Microarray Results*

The DNA microarray hybridization data suggested that *CYP1A1* and *CYP1B1* constitute the only non-estrogenic tran-

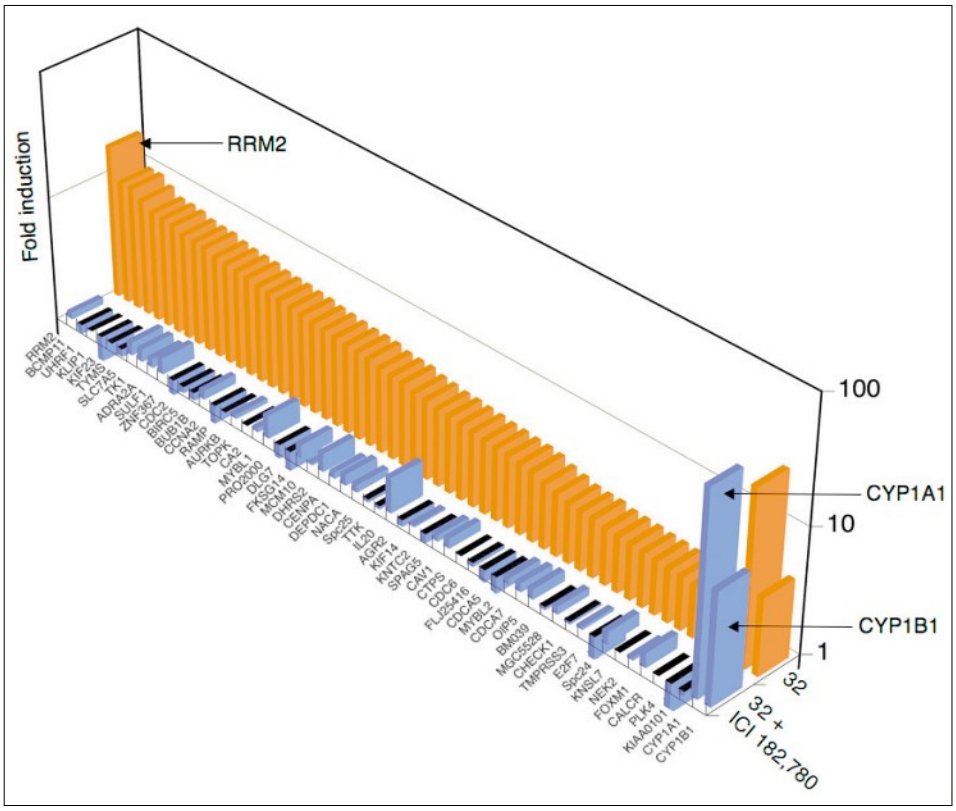


Fig. 4. Effect of an estrogen receptor antagonist (mean values of triplicate experiments). MCF7 cells were incubated with extract no. 32 in the absence (orange bars) or in the presence (blue bars) of ICI 182,780 (100 nM). The fold change is shown for the same 57 transcripts selected in Fig. 2. The effects of ICI 182,780 were calculated using, as the baseline, control experiments with 100 nM of the inhibitor alone. The fold induction values along the Y-axis are plotted in a logarithmic scale. Similar results (suppression of all transcripts except *CYP1A1* and *CYP1B1*) were obtained with extract no. 43 and 45.

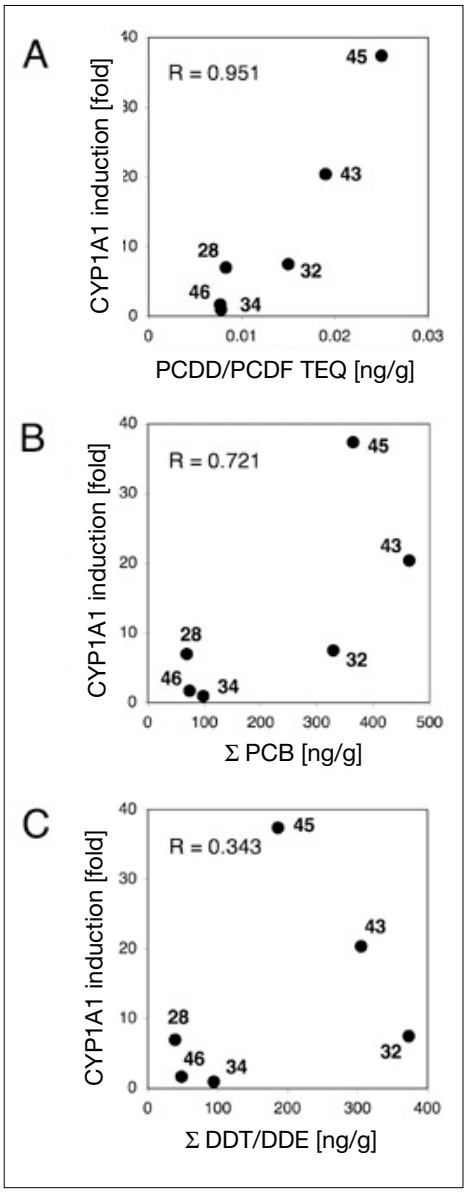


Fig. 5. Real-time RT-PCR validation of *CYP1A1* induction. The fold changes were determined in triplicate PCR assays and plotted against the respective concentrations of PCDDs/PCDFs (A), PCBs (B) or DDT/DDE (C). The correlation coefficients (R) were calculated assuming a linear relationship.

scripts in MCF7 cells that are increased after treatment with the breast milk extracts. To confirm this finding, real-time reverse transcriptase-PCR (RT-PCR) assays were carried out using specific primers. After normalization with the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control, all expression values were transformed as the ratio of messenger levels between treated cells and the solvent controls. The subsequent comparison of microarray hybridization data with the respective RT-PCR values showed a high degree of concordance. For the *CYP1A1* transcript, these quantitative PCR determinations yielded a 7.5- to 37.4-fold upregulation after incubation of MCF7 cells with the more contaminated samples no. 32, 43 and 45. In the presence of the less contaminated reference extracts no. 28, 34 and 46, the *CYP1A1* transcript was induced, on the average, only 3.2-fold. Moreover, these RT-PCR quantifications confirmed that the *CYP1A1* induction correlates well with the respective concentrations of PCDDs and PCDFs (Fig. 5A) and, to a lesser extent, with the concentration of PCBs (Fig. 5B). As expected, there was no relationship between *CYP1A1* induction and the contamination with DDT/DDE (Fig. 5C). The *CYP1B1* transcripts, determined by real-time RT-PCR, reached a 5.1-fold induction level and, as it was the case for *CYP1A1*, correlated with the presence of PCDDs/PCDFs and PCBs, but not with DDT/DDE (data not shown).

### 2.4. *Analysis by Reporter Gene Assay*

A key player in the induction of CYP enzymes by xenobiotics is the aryl hydrocarbon (Ah) receptor.[30] Thus, a reporter gene assay was performed to confirm that the Ah receptor pathway is stimulated in the presence of extracts prepared by gel permeation chromatography. For that purpose, each residue mixture obtained from the lipid component of individual milk samples was used to expose stably transfected H4IIE cells. This rat hepatoma cell line carries a chromosomally integrated construct that drives expression of the firefly luciferase gene under control of a minimal promoter flanked by dioxin response elements.[31] The Ah receptor activation was monitored after 24 h treatments by measuring the luciferase activity in cell lysates. The results of this bioassay, expressed as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) equivalents in the lipid fraction, ranged from 73 pg/g in sample no. 34 to 229 pg/g in sample no. 45. Thus, the reporter gene assay confirmed that all tested milk samples contained environmental contaminants in sufficiently abundant concentrations to activate the Ah receptor.

## 3. Discussion

Previous epidemiologic studies point to a role of *CYP1A1* polymorphisms in modulating the predisposition to breast cancer in postmenopausal women. At least two different genotypic variants of this metabolic enzyme have been associated with cancer risk in women carrying high body burdens of PCBs.[1,3,17]

*CYP1A1* is involved in the biotransformation of endogenous or exogenous compounds to yield genotoxic metabolites that cause DNA damage and mutations.[32–34] Quinone derivatives produced by cells of the breast epithelium are an example of genotoxic molecules resulting from the oxidation of endogenous hormones.[35] There is evidence that polymorphic variants result in a more inducible *CYP1A1* gene[36] and greater biotransformation activity would enhance the metabolic activation of procarcinogens, thereby increasing the risk of contracting breast cancer.[32,33]

PCBs have been shown to induce the *CYP1A1* gene in various experimental systems.[30] However, it was not known whether chlorinated compounds sequester in the human body to reach sufficiently elevated concentrations to be able to stimulate the expression of *CYP1A1* or any other relevant gene in the mammary gland. *CYP1A1* activity may be determined for example in peripheral blood lymphocytes, but these measurements are susceptible to large individual variations not related to particular genotypic differences or exposure levels.[37] As a consequence, the purpose of this study was to examine whether environmental contaminants released from the human adipose tissue are able to induce significant changes of gene expression in specific target cells. We took advantage of the fact that the lipid fraction of breast milk obtained from nursing mothers reflects the internal burden of persistent pollutants in adipose tissues.[6]

One of the best models to study the simultaneous effects of Ah and estrogen receptor ligands is the MCF7 cell line, which expresses both Ah and estrogen receptors.[22] Also, MCF7 cells were selected for this study because they originate from the mammary gland, where epithelial cells become exposed to the contaminants released from the adipose tissue or milk fat. We processed the lipid fraction of milk samples by gel permeation chromatography to isolate representative mixtures of a wide spectrum of contaminants including PCBs, PCDDs, PCDFs, DDT, DDE and PBDEs, along with endogenous hormones or metabolites. Our large-scale transcriptomic analysis demonstrated that these complex lipophilic extracts induce a predominantly estrogenic response in mammary cells that is fully attributable to the presence of the endogenous 17β-estradiol (Fig. 2). In fact, 17β-estradiol is transferred across the mammary gland generating biologically active concentrations in breast milk[38] and physiologic estrogens also appear in the lipid fraction.[39] According to our own measurements by liquid chromatograpyh/tandem mass spectrometry, the final 17β-estradiol concentration in the cell culture medium, due to its presence in milk fat, exceeded the *in vitro* $EC_{50}$ value of 6 pM. Thus, the observed estrogenic expression profile is induced by the endogenous hormone rather than exogenous factors such as, for example, pesticides or dietary phytoestrogens.

The only non-estrogenic responses arising from the exposure of MCF7 cells to breast milk extracts consisted of the induction of transcripts coding for *CYP1A1* and *CYP1B1*. Unlike all other transcriptional changes, the level of these two messengers was not suppressed by co-incubation with an estrogen receptor antagonist (Fig. 4). Dioxins and PCBs are potent CYP inducers and act mainly through Ah receptor activation.[18,40] This intracellular receptor undergoes heterodimerization upon ligand binding and, after translocation into the nucleus, binds to cognate *cis*-acting sequences located in the 5'-regulatory region of target genes such as those coding for major xenobiotic metabolizing enzymes. Two independent observations confirm that the lipophilic mixtures isolated from breast milk act through this pathway. First, the DR-CALUX reporter assay, based on a synthetic promoter that detects Ah receptor ligands in a highly selective manner,[31] proves that the Ah receptor is activated following treatment with breast milk extracts. Second, we observed that the amplitude of *CYP1A1* and *CYP1B1* induction, determined by real-time RT-PCR, correlates with the total concentration PCDD/PCDF (Fig. 5A) and PCBs (Fig. 5B).

The molecular consequences of combined mixtures of dioxins, pesticides and endogenous hormones are poorly understood.[22] To our knowledge, this is the first report where a large-scale transcriptomic study has been conducted to monitor the biological activity of complex substance mixtures encountered in the human body. Such a large-scale expression analysis appears to be particularly useful for the evaluation of mixture effects because it provides a suitable method to detect simultaneously the activation or repression of separate pathways. Interestingly, we observed a trend of decreasing estrogenic activity in the more heavily contaminated samples (Figs. 3B, 3C and 3D), suggesting that the incurred mixtures of chlorinated compounds may exert an overall anti-estrogenic activity, as noted before by Pliskova *et al.*[41] when analyzing the impact of chlorinated organic pollutants in the serum of male volunteers. This view is supported by the known anti-estrogenic activity of dioxins and dioxin-like chemicals.[19] Thus, the results of our whole-mixture approach is consistent with the extracts being able to induce *CYP1A1* and *CYP1B1* and, at the same time, suppress estrogenic responses.

The source of the detected organochlorines and the impact of smoking or other lifestyle factors in determining the exposure level of the different subjects of this study are unknown. Nevertheless, to our knowledge, this is the first proof that a mixture of contaminants accumulating within individual breast tissues is able to trigger the transcriptional induction of *CYP1A1* and *CYP1B1* in cells derived from the mammary epithelium. Our results provide a missing molecular link to explain the role of *CYP1A1* polymorphisms in the association between cancer risk and an elevated level of chlorinated chemicals. Based on our findings, it is likely that the endogenous burden of chlorinated chemicals leads to the induction of *CYP1A1*, thus promoting the formation of genotoxic intermediates or other hazardous metabolites. This cellular reaction may be enhanced in the presence of *CYP1A1* variants that are more susceptible to the transcriptional stimulus exerted by chlorinated compounds.[36,42] Conversely, the lack of significant associations between these chemicals and breast cancer in some studies (reviewed by Laden *et al.*[16]) is likely due to the fact that only particular genetic risk groups respond with a strong *CYP1A1* induction.

## 4. Experimental

### 4.1. *Standards*

All analytical standards were from the Cambridge Isotope Laboratories (distributed by LGC Promochem, Wesel, Germany); 17β-estradiol was purchased from Fluka (Buchs, Switzerland); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from the NCI Chemical Carcinogen Reference Standard Repository. The inhibitor ICI 182,780 was purchased from TOCRIS Bioscience (Avonmouth, UK).

### 4.2. *Human Breast Milk Samples and Extraction*

Breast milk samples (150–250 ml) from healthy nursing mothers, donated with informed approval between the 5th and 7th week after delivery, were stored at −20°C until use. The lipid fraction of each sample was separated by centrifugation and extracted as reported previously.[43] The lipids were removed by gel permeation chromatography on BioBeads S-X3 (Bio-Rad, Reinach, Switzerland) using cyclohexane/ethyl acetate as the solvent.

### 4.3. *Analysis of Extracts*

Cleanup procedures and gas chromatography/high resolution mass spectrometry (GC/HRMS) were based on methods described elsewhere.[43–45] Isotopically labeled analogs of each analyte of interest were used as internal standards.

### 4.4. *Calculation of Toxic Equivalents*

The relative potency of PCDD/PCDF was expressed as World Health Organization toxic equivalent (TEQ) concentrations.[46]

### 4.5. *Culture of MCF7 Cells and Treatments*

All cell culture media were from Gibco (Paisley, UK). The MCF7 cell line subtype BUS (provided by A.M. Soto and C. Sonnenschein, Tufts University, Boston, USA) was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). The antibiotics used were 0.1 U/ml penicillin and 0.1 µg/ml streptomycin. The cells were tested for the absence of *Mycoplasma* infection and cultured to 90% confluence at 37 °C in xenoestrogen-free plastic (Corning Inc., Grand Island, USA) under humidified air containing 5% $CO_2$. Before each exposure, the growth medium was replaced by phenol red-free DMEM and cells were cultured for 48 h in the presence of 5% charcoal/dextran-stripped FBS. Thereafter, the culture medium was reconstituted with the level of lipophilic contaminants that corresponds to breast milk with an approximate fat content of 5% (w/v). For that purpose, the residues obtained by gel permeation chromatography of 1.5 g milk fat were dissolved in 90 µl dimethyl sulfoxide (DMSO). Unless otherwise indicated, the resulting DMSO stock was diluted 333-fold in the cell culture medium to yield a final solvent concentration of 0.3% (v/v). Control cells were incubated with 0.3% (v/v) DMSO alone.

### 4.6. *Global Gene Expression Analysis*

After a 24 h incubation with milk extracts, MCF7 cells were collected by trypsinization and total RNA was recovered using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA quality was determined by analysis on the Agilent Lab-on-a-chip Bioanalyzer 2100. Samples with a total area under the 28S and 18S bands of less than 65% of the total RNA, as well as a 28S/18S ratio of less than 1.5, were considered to be degraded and therefore excluded from microarray analyses. Complementary DNA and RNA were synthesized using the cDNAsynthesis and IVT Labeling kits (Affymetrix, Santa Clara, CA), respectively. The biotin-labeled RNA was fragmented and hybridized on Human Genome U122 plus 2.0 microarrays (Affymetrix) following the manufacturer's instructions. After hybridization periods of 16 h, the microarrays were processed by automated washing on the Affymetrix Fluidics Station 450. Staining of the hybridized probes was performed with fluorescent streptavidin-phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried out on an Affymetrix scanner 3000. The data were normalized and analyzed using the GeneSpring 7.3.1 software (Agilent, Palo Alto, CA). The final results of triplicate experiments were imported into a Microsoft Excel file for graphical representation and determination of correlation coefficients.

### 4.7. *Real-time PCR Assays*

PCR validations were carried out to confirm the DNA microarray hybridization results. Primers for the selected transcripts as well as TaqMan probes were obtained from Applied Biosystems (Foster City, CA). Briefly, 100 ng of complementary DNA were mixed with 100 nM of forward and reverse primers and supplemented with 25 nM TaqMan probe in a final volume of 25 µl. The reactions were performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in 45 cycles (95 °C for 15 sec, 60 °C for 1 min) after an initial 10 min incubation at 95 °C. The fold change in the expression of each gene was calculated as described,[47] using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as the endogenous control.

### 4.8. *Reporter Gene Assay*

Stably transfected H4IIE cells were cultured and the DR-CALUX assay was carried out following the standard operating procedure provided by BioDetection Systems (Amsterdam, The Netherlands). Briefly, rat hepatoma cells were seeded in microtiter plates (Nunc, Rochester, USA) with 0.1 ml medium. When a confluence of at least 95% was reached, 0.1 ml of medium containing milk fat extract was added to yield a final DMSO concentration of 0.8% (v/v). Solvent controls and a standard TCDD dose response were included on each plate. After 24 h exposures, cells were harvested, lysed and assayed for luciferase activity on a microplate luminometer (Dynex, Chantilly, USA). All values were corrected for background luciferase expression detected in the presence of solvent alone. The data obtained from the different TCDD dilutions were fitted to a sigmoidal curve ($y = a_0 / [1 + (x/a_1)\, a_2]$; y = measured activity, x = TCDD concentration, $a_0$ = maximum activity, $a_1$ = EC50, $a_2$ = slope factor) and luciferase activity induced by milk fat extracts was converted to TCDD equivalents by interpolation.

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