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EFFECTS OF FOOD CONTAMINANT MIXTURES ON PUTATIVE TARGETS OF ENDOCRINE DISRUPTERS EXPOSURE: AN INTEGRATED TOXICOGENOMIC-BIOMARKERS APPROACH

EFFETTI DI MISCELE DI CONTAMINANTI ALIMENTARI SU TARGET PUTATIVI DI ESPOSIZIONE A INTERFERENTI ENDOCRINI: UN APPROCCIO BIOMARKER-TOSSICOGENOMICO

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Index

SUMMARY

The Endocrine Disruptors (EDs) are a class of chemicals that may interfere with the endocrine system that plays a crucial role in maintaining the physiological homeostasis of the human body as well as in regulating body growth, metabolism, reproduction and behavior (Faroon et al., 2001; Schell and Gallo, 2010). The EDs can exert their effects through a number of different mechanisms in particular interacting with nuclear (NRs) and orphan receptors, enzymatic pathways involved in steroid biosynthesis and detoxification metabolism (Diamanti-Kandarakis, 2009).

Polychlorinated biphenyls (PCBs) form a group of fat soluble and environmentally persistent EDs able to bioaccumulate in lipid fraction of animal tissue leading to biomagnification in the food chain. PCBs intake via food of animal origin represents the main route of human exposure (EFSA, 2010).

PCBs can be divided into two groups according to their biochemical and toxicological properties: Dioxin-like (DL) PCBs, mainly interacting with the Aryl Hydrocarbon Receptor (AhR) and Non Dioxin-like (NDL) PCBs whose mechanisms of action are not fully clarified. Actually, organisms are exposed to several congeners at the same time and, accordingly, the observed toxicological effects, such as on liver, thyroid, immune function, reproduction and behavior as well as carcinogenicity (EFSA, 2005), are exerted by mixtures. Therefore, new researches are required to study EDs mixtures, in particular on possible additive/synergistic effects at "real-life" dose levels.

In this study, 21 environmentally relevant PCB congeners were divided in three groups according to the proposed classification of Wolff et al. (1997) and Negri et al. (2003), based on structural and modes of action similarities: Mix1 (PCB 44, 49, 52, 101, 174, 177, 187, 201), potentially estrogenic NDL congeners, Mix2 (PCB 77, 81, 105, 114, 118, 126, 169) featuring DL-PCB and Mix3 (PCB 99, 153, 180, 183, 196, 203), NDL-PCBs highly persistent in the organism and phenobarbital-like inducers of Cytochrome P450 (CYP450).

The aim of the present project was to evaluate potential effects on liver and on adipose tissue as target organs of PCBs, elicited by the mixtures, at realistic human exposure levels, as derived from human exposure data through the evaluation of biomarkers response.

Two *in vitro* models have been selected as representative of liver tissue and corresponding to two different life stages: infant (HuH6) and pubertal (HepG2) cells.

To evaluate synergic effect, biomarkers of effect were used as indicators to measure PCB-induced variation in cellular or biochemical processes (NRs, NRF2, CYP450 enzymes and markers of oxidative stress). Moreover gene expression profiles of PCBs treated HepG2 cell line were evaluated by microarray analysis.
Moreover, PCBs bioaccumulation and permanence in animal fat could affect preadipocyte programming and differentiation and adipocytes metabolism. Therefore, differentiation of mouse (3T3-L1) and human preadipocytes (SGBS cells) was evaluated to determine the effect of PCBs exposure. Moreover gene expression of biomarkers involved in adipocytes differentiation and metabolism were analyzed in 3T3-L1.
Previous reports classified Mix1 as potentially antiandrogenic congeners (Wolff et al., 1995), on the contrary, we observed in HepG2 cell line, that Mix1 significantly up-regulated only AR suggesting a potential androgenic effect. In HepG2 cells, we also observed an uncoupling in ROS-CYP1A1 regulation and in HuH6, GSH/GSSG ratio was significantly higher. As for Gene Ontology enrichment analysis, Mix1-modulated gene list was uniquely significant for terms related to Nuclear Transport and significantly affected only one pathway, the Intestinal Immune Network for IgA production, implying a stimulation of the immune response. Overall, these molecular processes may be considered specific markers of Mix1 effect in hepatic cells.
As regards effects on adipocytes, Mix1 increased SGBS differentiation at 3x concentration and in 3T3-L1 affected only C/EBPα involved in adipose tissue development.
Mix2, featuring DL-PCB congeners, exerted the up-regulation of AR and AhR in HepG2 cells. Moreover, Mix2 increased ROS levels as well as CYP1A1 mRNA expression both mediated by AhR (Kopf et al., 2010).
However Mix2 significantly decreased the CYP1A1 enzyme activity, indicating a different effect possibly due to the low concentrations used.
In HuH6, Mix2 induced a down regulation of CAR mRNA expression that may explain the reduction of CYP1A1 and CYP3A4 activity. Therefore, CAR gene expression and CYP1A1 and CYP3A4 activities may be assumed as target of DL-PCBs disruption on this hepatic cell line.
Microarray results evidenced Mix2 affected genes involved in RNA processing and splicing, protein localization and catabolism and macromolecules degradation. Noteworthy, main and characteristic effects of Mix2 relied a) on enrichment of genes related several cancer pathways as p53, activator of genes involved also in cell cycle arrest (Lanni and Jacks, 1998), apoptosis and in communication in adjacent cells, and b) on cell

cycle progression, inducing of G1 phase and inhibiting S phase. So we may hypothesize that a sum of contrasting mode of action exerted by PCB congeners in Mix2 occurred.
As regards effects on adipocytes, Mix2 significantly induced SGBS differentiation only at 3x concentration, probably due to the mixture effect and/or the lower concentration levels used in this study.
Mix3 treatment affected the wider number of NRs expression (ERα, ERβ, AR, PXR, RARα, THRα) as well as NRF2, in particular, in HuH6. To our knowledge, it is the first study that showed AR mRNA expression due to PCB congeners included in Mix3, whereas the observed ERα and ERβ induction confirmed the estrogenic effect of PCB 99 and 153 (Warner et al, 2012).The decrease in the CYP3A4 enzyme activity in HuH6 cells was in agreement with NRF2 gene expression down-regulation (Itoh et al, 1997).
Microarray analysis showed that Mix3 modulated the higher number of genes (5979 genes) with 1501 shared with Mix2-affected genes. Apart some GO enriched terms related to cell organization, Mix3 did not share other effects with Mix1, the other NDL mixture.
One of the most interesting evidence is the enrichment of genes involved in the Wnt signaling pathway by only Mix3, suggesting an unbalance toward pluripotency promotion (Takemarua and Moona, 2000).
Mix3 also affected some cancer pathways as well as the adherens junction pathway as Mix2 but modulating a different panel of involved genes.
Moreover Mix3 exerted the higher magnitude of effect on pre-adipocytes differentiation in both murine and human cell lines. Gene expression evaluation analysis outlined that Mix3 down regulated Hes1, a DNA binding protein whose expression blocks adipogenesis (Ross et al., 2006).
Overall results confirmed that the adopted grouping of PCBs in three mixtures served to highlight different modes of action as well as biomarkers responses, also among mixtures featuring NDL congeners. Moreover, the two hepatic cell lines and the two pre-adipocytes cell lines appear to be differently reactive to PCBs, indicating the need to use different *in vitro* models and a panel of biomarkers in order to characterize the EDs effects.
These represented new results since there are no studies on PCB mixtures summarizing effects on oxidative stress, metabolism, nuclear receptors gene expression and adipocytes differentiation responses following treatment at human real exposure concentrations.
As prompted by EFSA (2005), it is necessary to provide evidences concerning comprehensive toxicological end-points of NDL effects. Therefore, these data may serve as a basis for developing relative toxicological factors for the NDL congeners risk assessment.

RIASSUNTO

Gli interferenti endocrini (IE) sono una classe di sostanze chimiche che possono interferire con il sistema ormonale. Il sistema endocrino svolge infatti un ruolo cruciale nel mantenimento della omeostasi fisiologica, nella regolazione della crescita, nel metabolismo, sviluppo e comportamento (Faroon et al., 2001). Gli IE possono esercitare i loro effetti attraverso una serie di meccanismi diversi come l'interazione con recettori nucleari (NR), con pathway enzimatiche coinvolte nella biosintesi degli steroidi, nel metabolismo e nella detossificazione (Diamanti- Kandarakis, 2009).
I policlorobifenili (PCB) costituiscono un gruppo di IE liposolubili e persistenti nell'ambiente. Negli organismi, hanno la caratteristica di bioaccumulare nella frazione lipidica dei tessuti con conseguente biomagnificazione nella catena alimentare. L'esposizione umana avviene principalmente attraverso il consumo di alimenti di origine animale (EFSA, 2010). I PCB possono essere suddivisi in due gruppi in base alle loro proprietà biochimiche e tossicologiche: PCB diossina-simili (DL), che interagiscono principalmente con il recettore arilico (AhR) e PCB non diossina-simili (NDL), i cui meccanismi di azione non sono completamente chiariti. In realtà, gli organismi sono esposti contemporaneamente a diversi congeneri presenti nei cibi e gli effetti tossicologici osservati sono quindi imputabili a miscele di PCB che possono esercitare una varietà di effetti su fegato, tiroide, immunità, riproduzione cosi come il cancro (EFSA, 2005).
Nuovi studi sono quindi necessari per evidenziare i possibili effetti additivi o sinergici di miscele di IE, in particolare a livelli di 'dose reale' per l'esposizione umana.
In questo studio, 21 congeneri di PCB di rilevanza ambientale sono stati divisi in tre gruppi sulla base di somiglianze strutturali e modalità di azione seguendo la classificazione proposta di Wolff et al. (1997) e Negri et al. (2003): Mix1 (PCB44, 49, 52, 101, 174, 177, 187, 201), che include congeneri NDL potenzialmente estrogenici, Mix2 (PCB77, 81, 105, 114, 118, 126, 169) che include DL-PCB e Mix3 (PCB99, 153, 180, 183, 196, 203) che contiene congeneri altamente persistenti nell'organismo umano e induttori dei citocromo P450 (CYP450).
Lo scopo del presente progetto è stato quindi quello di valutare i potenziali effetti provocati da miscele di congeneri di PCB di rilevanza ambientale sul fegato e sul tessuto adiposo, a livelli realistici di esposizione nell'uomo, in quanto derivati da dati umana misurati nella popolazione generale italiana.
Sono stati selezionati due modelli *in vitro* rappresentativi del fegato, tessuto bersaglio dei PCB e principalmente e coinvolto nel metabolismo,

corrispondenti a due diverse fasi della vita: bambino (HuH6) e adolescente (HepG2). Per valutare l'effetto sinergico delle miscele di PCB, sono stati utilizzati biomarcatori di effetto (NR, NRF2, enzimi CYP450 e marcatori di stress ossidativo) associabili ad una possibile variazione nei processi cellulari o biochimici indotta dall'esposizione a PCB; inoltre, nelle HepG2, è stato valutato il profilo di espressione genica mediante analisi microarray.
Ancora, lo studio esamina gli effetti dei PCB sul tessuto adiposo addominale poiché la permanenza dei PCB nella frazione grassa potrebbe influenzare il metabolismo degli adipociti e il programming e la differenziazione dei preadipociti. E' stato quindi valutato il tasso di differenziamento in fibroblasti di topo (3T3-L1) e preadipociti umani (SGBS) e, nelle 3T3-L1 esposte alle tre miscele, è stata valutata l'espressione genica di biomarcatori coinvolti in questi processi.
Precedenti studi classificano i congeneri della Mix1 come potenzialmente antiandrogeni (Wolff, 1995), al contrario, i nostri risultati evidenziano che Mix1 incrementa significativamente l'espressione di AR suggerendo un effetto androgenico. Nelle cellule HepG2, abbiamo anche osservato una regolazione della coppia di marcatori ROS-CYP1A1 e, in HuH6, il rapporto GSH/GSSG era significativamente più alto. Per quanto riguarda lo studio del profilo genico, si è evidenziato che l'elenco di geni modulati da Mix1 è significativo per termini relativi al trasporto nucleare e risulta significativamente influenzato solo il pathway del Network immunitario intestinale per la produzione di IgA che implica una stimolazione della risposta immunitaria. Nel complesso, questi processi molecolari possono essere considerati marker specifici di effetto di Mix1 nelle cellule epatiche.
Per quanto riguarda gli effetti sugli adipociti, Mix1 induce il processo di differenziazione nelle SGBS alla concentrazione 3x e nelle 3T3-L1 risulta modulata solo l'espressione genica di C/EBPα, coinvolto nello sviluppo del tessuto adiposo.
Mix2 (DL-PCB) determina una regolazione positiva di AR e AhR nelle HepG2. Inoltre, risulta incrementare i livelli di ROS nonché l'espressione del CYP1A1 entrambe AhR-mediati (Kopf et al., 2010). Tuttavia l'attività dell'enzima CYP1A1 è significativamente diminuito, indicando un effetto contrastante probabilmente dovuto alle basse concentrazioni utilizzate.
In HuH6, Mix2 regola negativamente l'espressione di CAR che potrebbe spiegare la riduzione del CYP1A1 e del CYP3A4; questi tre parametri possono essere assunti come marcatori specifici dei DL-PCB su questa linea cellulare epatica. L'analisi Microarray evidenzia per Mix2 la modulazione di geni coinvolti nel processamento e splicing del RNA e nella localizzazione delle proteine. Degni di nota sono inoltre gli effetti su geni

correlati al cancro come la p53, coinvolta nell'arresto del ciclo cellulare (Lanni e Jacks, 1998), apoptosi e nella comunicazione cellulare ma anche di fattori legati alla progressione del ciclo cellulare, per cui si può ipotizzare un equilibrio fra modi di azione contrastanti esercitati dai congeneri di PCB in Mix2. Per quanto riguarda gli effetti sugli adipociti, Mix2 induce la differenziazione nelle SGBS a concentrazione 3x, probabilmente per l'effetto miscela o delle concentrazioni utilizzate.
Per quanto riguarda la Mix3, il trattamento influenza l'espressione di un ampio numero di NR (ERα, ERβ, AR, PXR, RARα, THRα) nonché NRF2 nelle HuH6. Questo è anche il primo studio che ha dimostrato un effetto sull'espressione di AR dovuto ai congeneri della Mix3, mentre l'induzione osservata di ERα e ERβ conferma l'effetto estrogenico dei PCB99 e 153 (Warner et al, 2012). Inoltre la diminuzione dell'attività del CYP3A4 in HuH6 è in accordo con la down regolazione di NRF2 (Itoh et al, 1997). L'analisi Microarray di Mix3 mostra la modulazione del più alto numero di geni (5979), 1501 dei quali condiviso con Mix2 mentre, con l'altra miscela di NDL, non ha effetti comuni se non quelli relativi all'organizzazione cellulare. Uno degli elementi più interessanti è comunque l'arricchimento di geni implicati nella pathway del signaling di Wnt, modulati esclusivamente da Mix3, che suggerisce la promozione verso la pluripotenza (Takemarua e Moona, 2000).
Mix3, influenza anche alcuni pathway implicati nel cancro nonché, come Mix2, nelle giunzioni aderenti, rispetto alla quale modula un diverso gruppo di geni coinvolti. Inoltre la Mix3 esercitava gli effetti maggiori sulla differenziazione dei preadipociti sia nella linea cellulare umana che in quella murina e l'analisi dalla modulazione genica evidenzia che reprime Hes1 la cui espressione blocca l'adipogenesi (Ross et al., 2006).
Complessivamente i risultati hanno confermato che la divisione dei PCB in tre miscele individua diverse modalità di azione, nonché differenti risposte dei biomarcatori, anche tra miscele di congeneri NDL. Inoltre, le quattro linee cellulari (epatiche e di preadipociti) sembrano essere diversamente sensibili ai PCB, ciò indica la necessità di utilizzare diversi modelli *in vitro* e un pannello di biomarcatori per caratterizzare gli effetti degli IE.
In conclusione, tutti questi rappresentano nuovi risultati data l'assenza di dati sugli effetti dell'esposizione a miscele di PCB a 'concentrazioni reali' che includano stress ossidativo, metabolismo, espressione dei NR e differenziazione degli adipociti. Come sottolineato dall'EFSA (2005), vi è la necessità di dati riguardanti end-point tossicologici dei NDL, pertanto, questi dati rappresentano un contributo utile alla caratterizzazione di fattori tossicologici per la valutazione del rischio legato ai NDL-PCB.

BACKGROUND

Endocrine disruptors

Endocrine disruptors (EDs) are a diversified class of chemicals that interfere with the hormonal system. The term *"endocrine disrupter*" was introduced in the early 1990s and later defined by the World Health Organization (European Commission, 1996) as: *"… an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"*.

The endocrine system plays a crucial role in maintaining the physiological homeostasis of the human body, in regulating body growth, metabolism, sexual development and function as well as reproduction (Faroon et al., 2001), therefore an impingement on these processes by EDs may severely affect human health.

EDs include persistent contaminants, (e.g. dioxins, Polychlorinated Biphenyls –PCBs-, Polybrominated Diphenyl Ethers -PBDEs), pesticides (organophosphates, di-thiocarbamates), plasticizers (e.g. phthalates, bisphenol A) as well as natural compounds (e.g. phyto-oestrogens which are present in plants such as soy and nuts, coumestrol and genistein) or pharmaceuticals (such as diethylstibestrol, 17a-ethinyloestradiol and tamoxifen). EDs are widespread in food and feed, being primary routes of exposure for both synthetic (man-made) and natural EDs, therefore representing a great concern for human and wildlife health. In particular, lipophilic and persistent compounds may bioaccumulate in the lipid fraction of animal tissue leading to a biomagnification in the food chain. Therefore, fatty fish, meat and dairy products are among the major sources of some persistent EDs (EFSA, 2012).

Due to their different chemical structures, EDs as DDT, bisphenol-A and nonylphenol (Eisenbrand, 1996; Shafer et al., 1996) may exert multiple effects. Indeed, the endocrine and reproductive effects of these chemicals are related to their ability to interact with nuclear receptors (NRs) by: (a) mimicking the effect of endogenous hormones; (b) antagonizing the effect of endogenous hormones; (c) disrupting the synthesis and metabolism of endogenous hormones; (d) disrupting the synthesis of hormone receptors.

The biological actions of endogenous hormones, such as estrogen, progesterone, testosterone and thyroxine, are mediated by the binding with high-affinity receptors located into target cells, the NRs. The interaction of

an hormone with its receptor initiates a cascade of events leading to multiple effects depending on that particular hormone (Amaral Mendes, 2002). Originally, EDs were thought to exert primary actions only through NRs, mainly steroid ones, such as estrogen receptors (ERα and ERβ), androgen receptor (AR), progesterone receptor (PR), thyroid receptors (THRα and THRβ), and recent evidence indicates that other endocrine pathways are also involved by the disrupting chemicals as retinoid receptors (RARs and RXRs). Growing evidence showed that the mechanisms involved are broader since EDs may interact also with nonnuclear steroid hormone receptors (e.g., membrane ERs), nonsteroid receptors (e.g., neurotransmitter receptors such as the serotonin, dopamine and norepinephrine receptors), orphan receptors (e.g., Aryl hydrocarbon Receptor (AhR), through numerous other mechanisms converging upon endocrine and reproductive systems (Diamanti-Kandarakis, 2009).
Moreover, it is increasingly evident that many EDs may act as agonists in some tissues and as antagonists in others. Indeed different tissues express co-activators or co-repressors that could modulate the ligands-action in a tissue-specific manner implying that EDs may regulate same pathways but in opposite ways.
Such complexity in the mechanisms of action imply different adverse effects may occur depending on the chemical, the exposure concentration and the individual susceptibility. Critical stages of development (infants, children and unborn babies) may be particularly sensitive to EDs hormonal activity, especially during defined "windows of susceptibility". Exposure of pregnant women to EDs may increase the likelihood of toxic effects in fetus possibly resulting in abnormalities or diseases in the short-term or evident only later in life (Swan et al., 2005; Castro et al., 2008; Deckelbaum and Williams, 2001).

PCBs, a class of Endocrine disruptors

Among EDs, Polychlorinated biphenyls (PCBs) are a class of chemicals widely used in the past for many applications, especially as dielectric fluids in transformers, capacitors and coolants. Due to their toxicity and classification as persistent organic pollutants, processing and distribution of PCBs has been prohibited in industrial countries since the late 1980s but they still can be released into the environment from building paint and sealants or poorly maintained hazardous waste sites containing PCBs. Depending on the number of chlorine atom substituents (1-10) and their

position on the two aromatic rings, 209 possible congeners may be synthesized.
PCBs are widely present in the environment due to a global circulation by atmospheric transport. Highly chlorinated congeners, in particular, adsorb strongly to sediment and soil, where they tend to persist with half-lives of months to years.
Due to their lipophilicity, PCBs tend to bioaccumulate in body fat, both in animals and humans, biomagnifying through the food chain; so exposure to PCBs occurs mainly through the diet and it is especially related to certain food commodities, such as dairy products and fatty fish (EFSA, 2010).
This is particularly the case for higher chlorinated congeners, while lower chlorinated PCB congeners are metabolized quickly.
Following ingestion, PCBs are mainly biotransformed in the liver to hydroxy (HO-) and methylsulfone (MeSO2-) metabolites.
PCBs exert a wide spectrum of adverse effects, especially at neurochemical and neuroendocrine level (Tilson and Kodavanti, 1997), as well as reproductive level. An association between endometriosis and high levels of PCB in plasma has been reported since 1992 (Gerhard and Runnebaum, 1992), moreover a relationship was found with long-term aspects of Testicular Dysgenesis Syndrome such as low sperm count and testicular cancer (Hardell et al., 2003, 2004; Toppari et al., 1996).
Prenatal treatment of female rodents and other species with PCBs has been shown to alter the timing of puberty (Humblet et al., 2011), accelerate reproductive aging (Cooper and Kavlock, 1997), affect gonadotropin release (Khan and Thomas, 1997), and interfere with sexual behavior (Chung and Clemens, 1999). There is evidence that the PCBs can affect thyroid hormone status; indeed Schell and Gallo (2009) study showed that PCB levels are positively related to TSH and negatively to free T4.
PCBs have been classified as possible human carcinogens (WHO-IARC, 1987). Several studies have implicated low-molecular-weight PCBs as potential tumor-initiating compounds that may either induce oxidative DNA damage or formation of DNA adducts (Oakley et al., 1996a, 1996b). Several case-control studies, published in the last two decades, have raised the issue that women, exposed to organochlorine chemicals such as DDT and certain PCB congeners, may have a higher incidence for breast cancer than non-exposed women (Cohn et al., 2012).

PCB congeners classification

From the 209 theoretically possible PCB congeners, 12 non-ortho and mono-ortho substituted PCBs show toxicological properties similar to dioxins, and therefore termed 'dioxin-like PCBs' (DL-PCBs), due to their coplanar structure and the capability to bind to the AhR receptor (Poland et al., 1985; Safe, 1986; Safe et al., 2005; EFSA 2010). The 12 DL-PCBs also display anti-estrogenic activity in several biological systems (Letcher et al., 2002; Pliskova et al., 2005).

2,3,7,8-Tetrachlorodibenzo*p*- dioxin (TCDD), commonly used as the model compound of this large family of dioxin-like food contaminants, elicits a wide array of biochemical and pathological effects on mammals, including weight loss, hyperkeratosis, liver enlargement, thymus atrophy, immunosuppression, tumor promotion, impaired reproduction, and embryo toxicity (WHO, 1998).

In order to sum up and compare the toxicity of the different congeners of concern (17 dioxins and 12 dioxin-like PCBs), toxicity equivalency factors (TEF) have been developed on the basis of their relative toxicity compared to 2,3,7,8-TCDD (Van den Berg et al., 2006). The TEF approach is primarily intended for estimating exposure and risks. The other PCB, referred to as 'non dioxin-like PCB' (NDL-PCB), cannot adopt a coplanar structure so the TEF concept used for DL-PCBs can't be applied to NDL-PCB, moreover mechanisms of action of all congeners have not been evaluated yet. EFSA 2005. The 197 non dioxin-like PCBs (NDL-PCBs) (including 28 mono-ortho, di-ortho and more chloro-substitutedPCBs) are more abundant in the environment than DL-PCBs, with the highest mean contamination level observed in fish and fish-derived products followed by eggs, milk and their products, meat and meat products from terrestrial animals. Some NDL-PCBs elicit different types of responses including neurological, neuroendocrine, endocrine, immunological and carcinogenic effects. These effects occur via multiple toxicity pathways, not involving AhR (EPA, 2003). Six congeners (28, 52, 101, 138, 153, and 180) were chosen as indicators because their sum represent about 50% of the total NDL-PCBs in food. Moreover, they mostly contribute to the anti-androgenic, (anti)estrogenic, anti-thyroidal, tumor-promoting, and neurotoxic potencies calculated for NDL-PCB mixtures in human samples As a consequence, the EU Commission recently established maximum levels for the sum of the six indicator NDL-PCBs in food and feed (Commission Regulation (EU) No 1259/2011).

PCB-168 is a congener not regularly monitored but it might significantly contribute to the AR-antagonistic and TTR-binding potency of PCBs complex mixture in human samples. Based on the current knowledge of

PCB levels in human tissues and toxic potencies of individual congeners, the seven indicators, PCBs plus PCB-168, may explain an average > 74% of the calculated toxic potency of PCB mixtures in humans (Hamers et al, 2011).
Many projects aim to clarify biological mechanisms underlying the various types of toxicity of NDL-PCBs and to evaluate these data from a regulatory toxicology point-of-view.
No published peer-reviewed data were available on the carcinogenic potency of single NDL-PCBs or about the potential mutagenic activity of PCB mixtures (EFSA, 2010). PCBs exposure occurs almost exclusively as a mixture of congeners having different effects. For this reason, some authors (Negri et al., 2003; Wolff et al., 1997) proposed a toxicologically based classification of PCBs in three groups, one DL and two NDL mixtures by introducing a distinction within the group of NDL-PCBs on the basis of structure-activity considerations. They named Group I, the estrogenic / neurotoxic and variably persistent congeners, with one or two unsubstituted para positions (PCB 44, 49, 52, 101, 174, 177, 187, 201) and Group III, the biologically persistent, phenobarbital (PB)-type cytochrome-P450 (CYP1A and CYP2B) inducing congeners (PCB 99, 153, 180, 183, 196, 203). Group II includes non-ortho, mono-ortho and di-ortho congeners with dioxin-like properties and potentially anti-estrogenic (DL-PCBs: PCB 77, 81,105, 114, 118, 126, 169). The partition of NDL-PCB may represent a possible way to differentiate and characterize the effects within the NDL-PCB group in order to define a proper toxicological significance.

Liver as PCBs target organ

The digestive system is exposed to contaminants and other food bioactive compounds, during all life stages, and such exposure is mediated by tissues extremely rich in different NRs. Indeed, tissues of the digestive system are highly responsive to endocrine regulation, and interference as well. Besides to be target of endocrine disruption, liver is the representative organ of the digestive system, hence involved in food processing and regulating fundamental steps of absorption and metabolism of food constituents.
Furthermore, liver is the organ involved in detoxification metabolism also of food contaminants, highly responsive to endocrine regulation, included PCBs. Biotransformations are thus critical processes in the body defense against the toxic effects of a wide variety of endobiotics and xenobiotics

(Yu, 2001). The biotransformation processes consist of three phases. Phase I includes activity of cytochrome P450 (CYP) super-family (heme-dependent mono-oxygenases) members expressed within the liver, intestine and kidney, the primary organs for uptake, metabolism, and excretion of xenobiotics (Kliewer et al., 2002). The CYP microsomal enzymes catalyze the metabolic conversion to more polar derivatives of foreign chemicals as well as endogenous substrates (e.g., steroid hormones) (Xu et al., 2005). Phase II includes synthetic or conjugation reactions, combining the toxicants or the primary metabolites directly with endogenous substances (e.g., Gly, Cys, glutathione). Often these processes result in the production of a more stable, water-soluble, metabolite that is more readily excretable via the transporter-mediated elimination pathway or phase III (Di Masi et al., 2009).

PCBs effects on liver cells

PCBs and nuclear receptors:

Estrogen receptors α and β

Estrogen receptors (ERs) belong to the steroid/thyroid hormone superfamily of NRs (Evans, 1988). In the nucleus, estrogen modulates gene transcription (genomic response), and the resulting protein products determine the cell biological actions of the sex steroid. In addition, a small pool of ERs localized to the plasma membrane, is involved in non-genomic response and signals mainly through direct or indirect coupling to G proteins (Marino et al, 2002). Therefore, in response to steroids binding, signal transduction modulates both non-transcriptional and transcriptional events and impacts both the rapid and more prolonged actions of estrogen. (Levin, 2005). Binding of a ligand to ERs triggers a series of conformational changes in the receptor structure and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes. These events include receptor dimerization, receptor-DNA interaction, recruitment of and interaction with co-activators and other transcription factors, and formation of a preinitiation complex (Nilsson et al, 2001).
There are two different forms of the ERs, α and β, encoded by separate genes (ESR1, estrogen receptor 1 (ER-alpha); ESR2, estrogen receptor 2 (ER-beta), respectively). Both ERs are widely expressed in different tissue

types, however with some notable differences in their expression patterns (Couse et al 1997).
Estrogens influence many physiological processes in mammals, including reproduction, cardiovascular system, bone integrity, cognition and behavior. DL-PCBs have been frequently reported to have anti-estrogenic activity, as TCDD (Buchanan et al. 2000, 2002; Oenga et al. 2004; Safe and Wörmke 2003), whereas the exact mechanisms of estrogenic or anti-estrogenic activities of NDL- PCBs are still not fully characterized.
The reported results are often contradictory, derived from data obtained in different *in vitro* or *in vivo* models (Hansen et al., 1998). While low molecular-weight PCBs displayed estrogenic activity both *in vitro* and *in vivo* (Arcaro et al., 1999; Nesaretnam and Darbre, 1997; Rogers and Denison, 2000; Rose et al., 2002), the three most prevalent NDL-PCBs, PCB 138, 153 and 180 have been reported to be anti-estrogenic in MCF-7 cells (Bonenfeld-Jorgensen et al., 2001). However, estrogenic/anti-estrogenic potencies of a large set of PCB congeners have not yet been determined in a single *in vitro* bioassay.

Aryl hydrocarbon receptor (AhR)

AhR is a member of the bHLH-PAS (basic helix-loop helix/Per-Arnt-Sim) gene family of transcription factors (Gu et al., 2000) and it is the only ligand-activated one. The majority of AhR ligands are environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), TCDD (Poland et al., 1976), related polychlorinated dibenzo-pdioxins (PCDDs) and dibenzofurans (PCDF) (Poland et al., 1982), and dietary indole carbinols present in cruciferous vegetables (Bjeldanes et al., 1991; Bradlow et al., 1991; Chen et al., 1998; Vang et al., 1991), so AhR acts as a toxin sensor.
After ligand binding, AhR migrates in the cell nucleus forming an heterodimeric complex with the AhR nuclear translocator Arnt (Hoffman et al., 1991) which binds to specific genomic enhancer sequences (up to 59) termed Dioxin- or Xenobiotic-Responsive Elements (DREs or XREs); this interaction leads to transcriptional activation of genes involved in complex cellular responses such as cell cycle progression and apoptosis such as phase I drug-metabolizing enzymes like *Cytochromes P-450* 1A1 (CYP1A1), CYP1A2, and CYP1B1 and phase II enzymes including glutathione-S-transferase Ya subunit, UDP glutathione transferase, aldehyde dehydrogenase, and NAD(P)H quinone oxidoreductase (NQOR)

(Hankinson et al., 1995). In addition, a number of Estradiol(E2) - regulated genes are controlled by AhR at either the transcriptional or post-transcriptional level (Nilsson et al., 2001).
NDL-PCBs are not documented to directly interact with AhR. Otherwise, similarly to TCDD, DL-PCBs activate AhR and AhR-dependent signal transduction pathways (Van Den Berg et al. 1998) which mediate the majority of the adverse effects of these compounds among which their anti-estrogenicity.
The anti-estrogenic action of AhR agonists such PCBs might involve repression of E2-dependent gene expression by interactions of activated AhR with DNA regions of E2 responsive gene promoters (Oenga et al., 2004; Safe and Wörmke, 2003), inhibition of E2-induced cell cycle proteins (Buchanan et al. 2002; Wang et al. 1998), or effects of PCBs on E2 metabolism (Pang et al., 1999; van Duursen et al., 2005).
AhR ligands are able to play their antagonistic effects on ER signaling by increasing the metabolism rate of E2 by inducing CYP1A1 and CYP1B1 enzymes (Hayes et al., 1996; Safe et al., 1991) or by decreasing levels of ERs.
Several genes have been identified as targets for cross-talk between ER and AhR: cathepsin D, pS2, prolactin receptor, c-fos, hsp27, TGF-a and TGF-b, as well as PR.

Androgen receptor

The androgen receptor (AR) is a member of the NRs superfamily, functions as a ligand-inducible transcription factor and is expressed in many end-organs including the hypothalamus, pituitary, liver, prostate, and testes (Matsumoto, 2008). The principle steroidal androgens, testosterone (T) and its metabolite 5-dihydrotestosterone (DHT), predominantly mediate their biological effects through binding to the AR.
The appropriate regulation of androgen activity is necessary for a range of developmental and physiological processes, particularly male sexual development and maturation, as well as the maintenance of male reproductive organs and of spermatogenesis (McLachlan et al., 1996; Mooradian et al., 1997; Cunha et al., 1987). The binding of T or DHT to AR induces receptor dimerization, translocation into the nucleus, binding to its cognate response elements (ARE) and recruit co-regulators to promote the expression of target genes (He, 1999; Quigley et at., 1998, Heinlein et al., 2002). In addition to this transcriptional or genomic mode of action by

steroids, androgens, like progesterone and estrogen, can exert rapid, non-genomic effects (Falkenstein et al., 2000) typically involving the induction of second messenger signal transduction cascades, including increases in free intracellular calcium, and activation of protein kinase A (PKA), protein kinase C (PKC), and MAPK. The non-genomic action of androgens has been implicated in a number of cellular effects, including gap junction communication, aortic relaxation, and neuronal plasticity (Pluciennik et al., 1996; Kubli-Garfias et al, 1982; Yamada et al, 1979; Costarella et al, 1996).
Dysregulation of androgen/AR signaling perturbs normal reproductive development and accounts for a wide range of pathological conditions such as androgen-insensitive syndrome, prostate cancer, and spinal bulbar muscular atrophy (Matsumoto, 2012).
Prenatal testosterone exposure alters placental steroidogenesis and leads to dysregulation of lipid metabolism in their adult female offspring. (Sun, 2012).
Endocrine disruptors that act via the androgen receptor (AR) are less well studied than environmental estrogens. Epidemiologic studies of occupational exposure to PCBs revealed a strong exposure response relationship for prostate cancer risk (Ritchie, 2003) and prostate cancer mortality (Prince et al, 2006) suggesting an anti-androgen mechanism for PCBs.

Thyroid receptor α (THRα)

THRs belong to the superfamily of hormone NRs. There are two major THR isoforms encoded on separate genes, designated as THRα and THRβ (Lazar et al, 1993).
The natural ligands of THRs are thyroid hormones (TH; thyroxin, T4; triiodothyronine, T3). THs play critical roles in differentiation, growth, and metabolism. Indeed, TH is required for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (Oppenheimer et al., 1987). THRs are target of endocrine disruption. Several reports have shown that PCBs may affect embryonic and neonatal development at low doses (Jacobson et al., 1990, 1996) causing abnormal development of the central nervous system (Jacobson et al., 1990) probably through the involvement of thyroid hormones system, since THs and PCB molecules share structural similarity (Koibuchi et al., 2000). Hypothyroidism during perinatal period causes retardation of general growth as well as abnormal development of many organs including brain,

bone, skeletal muscle and liver. Thus, disruption of TH system by PCB may affect development of such organs.

Peroxisome proliferator receptor γ

The nuclear Peroxisome Proliferator-Activated Receptors (PPARs) subfamily of NRs regulate the transcription of many genes in a ligand-dependent manner by binding to specific response elements (PPREs) within promoters. PPARs bind as obligate heterodimers with a Retinoid X Receptor (RXRα, β, γ) and, upon binding agonist, interact with cofactors increasing the rate of transcription initiation (Chawla et al., 2001). RXR–PPARγ complex can be transcriptionally activated by both PPARγ and RXR-specific ligands. PPARs are present in humans in three different isoforms, PPARα, PPARβ/δ and PPARγ that are encoded separately, but have overlapping tissue expression patterns, and as a group coordinate the regulation of important metabolic pathways (Sonoda et al., 2008; Chen et al., 1993; Lazar, 2005). PPARγ, the best-studied member of the family, results to be expressed at high levels in white and brown adipose tissue where it plays a major role in many different biological processes including lipid metabolism, glucose homeostasis, adipogenesis, lipid storage and release (Lehrke et al., 2005). Adipose tissue and large intestine have the highest levels of PPARγ mRNA; kidney, liver, and small intestine have intermediate levels. (Fajas, 1997). PPARγ is activated by endogenous arachidonic acid metabolites such as 15-deoxy-delta 12, 14 prostaglandin J2 (Forman et al., 1995). One class of PPARγ ligands, the thiazolidinediones, which includes the drug rosiglitazone, are effective insulin sensitizers, and have been shown to improve glucose uptake and lower hyperglycaemia and hyperinsulinaemia (Lehmann et al., 1995). The PPARs are also potential therapeutic targets for atherosclerosis, inflammation and hypertension (Berger et al., 2002) and tumor susceptibility (Lee et al., 2012).
Exposure to persistent organic pollutants, such as PCBs, may therefore contribute to the development of inflammatory diseases such as atherosclerosis. Cellular oxidative stress and imbalance in antioxidant status are critical events in PCB-mediated induction, contributing to endothelial inflammatory response in part by down-regulating PPAR signalling (Hennig et al., 2005).

Orphan receptors: Costitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR)

The large NRs subfamily I contains numerous orphan receptors including Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR). (Hustert et al., 2001).
CAR and PXR are modular proteins sharing common regions, including the N-terminal DNA-Binding Domain (DBD), the hydrophobic core region (H region), and the C-terminal Ligand-Binding Domain (LBD). (Olefsky, 2001). CAR-DBD and PXR-DBD are involved in receptor dimerization and binding to specific DNA sequences (Frank et al., 2003).
Although CAR and PXR sequences are closely related, their role in gene expression regulation is significantly different; indeed CAR and PXR recognize different endogenous and exogenous ligands. (Frank et al., 2003).
Due to their role in protecting living organisms from exogenous insults, CAR is highly expressed in the liver and in the epithelial cells of the small intestine villi (Swales and Negishi, 2004).
PXR is primarily expressed in liver, intestine, and kidney (Kliewer et al., 1998).
PXR takes part to a protective system to prevent exposure of critical cells sensitive to xenobiotics. A variety of compounds can activate both CAR and PXR. However, ligand-binding techniques indicate that CAR and PXR bind a structurally diverse panel of chemicals and the receptor ligand specificity is species-dependent. This promiscuity is warranted by the structural properties of CAR and PXR-LBDs to respond to a diverse set of low-affinity ligands (Lemaire et al., 2006;Kublbeck et al, 2011).
PXR seems to be much more promiscuous than CAR (Moore et al., 2003). It is now established that PXR binds nearly all xenobiotics including drugs such as dexamethasone, PCBs and nonylphenol (Lemaire et al., 2006). CAR activators promote the nuclear translocation of CAR by a ligand-dependent process, followed by CAR/RXRα heterodimerization, CAR-DBD binding to the RE, and recruitment of co-activators. CAR nuclear translocation not necessarily leads to an increase in target genes transcription but serves as the first important step to regulate the xenosensor transcriptional activity (Timsit and Negishi, 2007).
CAR and PXR function as sensors of toxic by-products of endogenous metabolic compounds and of exogenous chemicals to facilitate their elimination. Many mechanisms have been evolved to detoxify the majority of xenobiotics and versatile inducible metabolizing enzymes and efflux transporters play a crucial role in these mechanisms that are a form of biotransformation.
CAR coordinates the regulation of multiple hepatic genes resulting, in most cases, in metabolic detoxification by CYPs and transferases within the

hepatocyte. PXR serves as a master transcriptional regulator of CYP3A isozymes (Quattrochi and Guzelian, 2001). Indeed, PXR and CYP3A result co-expressed in several tissues; moreover PXR is activated by CYP3A inducers. (Di Masi et al, 2009).
It has been reported that PXR-mediated induction of CYP3A4 as well as CAR may be mediated by exposure to PCBs (Al-Salman et al, 2012) potentially causing adverse interactions. The OH group is commonly found in activators of PXR and CAR as exemplified by endocrine disrupters such as the hydroxylated PCBs. One of the most interesting differences involves highly chlorinated PCBs which were found to act as antagonists of PXR and agonists for rodent PXR (Lemaire eet al, 2004).
Two non-hydroxylated PCB congeners (PCB 118 and 153), which present a larger fraction of the PCB contamination of fatty foods, activated PXR (Jacobs et al, 2005).
Several of the non-coplanar PCBs directly activate PXR and CAR, whilst the coplanar PCB-77 did not. Non-coplanar PCBs were also able to activate PXR/CAR target gene expression in a substitution- and tissue-specific manner. Chronic activation of PXR/CAR is linked to adverse effects and must be included in any risk assessment of PCBs (Al-Salman et al., 2012).

Retinoic acid receptor α (RARα)

Retinoic acid receptors (RARs and RXRs) are ligand-dependent transcription factors, which belong to different subfamilies of NRs (I and II, respectively, according to the official nomenclature (NRNC, 1999). There are different RAR and RXR subtypes (α, β and γ) (De Lera et al., 2007).
RARα (NR1B1) is the most prevalent isoform of retinoic acid receptors in liver and hepatocytes. Two other receptors, RARβ and RARγ have complex tissue and developmental expression patterns.
RARs mediate the biological effects of all-trans-retinoic acid (at-RA) (Bastien et al., 2004), the most active naturally occurring retinoid in mammals, except for the visual process, where retinal is the active retinoid form. In the body, at-RA is formed from its precursor all-trans-retinol (at-ROL) (Duester et al., 2008; Pares et al., 2008). Retinoids (vitamin A and its analogues) play essential roles in several physiological processes, such as embryonic development, reproduction, immunity, proliferation, differentiation, apoptosis and vision (Luo et al., 2006; Niederreither et al., 2008; Kim et al., 2008). at-RA binds to the ligand-binding domain of RARs, which induces heterodimer formation with RXRs to form the

transcriptionally active complex. The heterodimer complexes act as transcriptional regulators of a multitude of retinoid-regulated genes by binding to specific RA response elements (RAREs) (Schuchardt et al., 2009).
Many genes involved in hepatic functions are regulated by RXR:RAR heterodimers. Among these are some involved in serum protein synthesis (a-fetoprotein, albumin, coagulation factors), bile acid synthesis (CYP7A1), and bile acid transporters (NTCP and MRP2) (Li et al., 2002). Environmental pollutants interfere with normal retinoid physiology and the change of retinoid levels in organism has been used as a sensitive biomarker of exposure to wide range of pollutants in wild animal populations. The liver seems to be an important target organ in dioxin disrupted Vitamin A status. (Hoegberg et al., 2005).
PCDDs, PCDFs and PCBs interfere with Vitamin A storage and metabolism in mammals, although the underlying mechanisms are not yet fully clarified (Nilsson et al., 2002).
Effects of TCDD on Vitamin A processing include an early increase in retinoid mobilization from retinyl ester stores (Kelley et al., 2000), altered retinol esterification (Hoegberg et al., 2003), altered tissue levels of retinoic acid, increased whole-body turnover of retinoids (Kelley et al., 1998), and increased excretion of polar retinoid metabolites (Brouwer et al., 1989). Secondly, metabolites of certain compounds like hydroxylated PCBs (Van Der Plas et al., 2001) may disrupt binding of retinoids to retinoid binding proteins. Mobilization of hepatic retinyl esters increases retinol and retinoic acid levels in serum (Hoegberg et al., 2003). Other described toxicity mechanisms could involve down-regulation of retinoic acid dependent growth factor TGF-β (Lorick et al., 1998) as observed in vitro with TCDD. While DL compounds may increase plasma retinol levels by decreased generation or increased mobilization of retinol storage forms, the total PCB load may deplete plasma retinol levels by disrupting RBP–TTR complex. Also an induction of CYPs (e.g. CYP2B and CYP3A) might be involved in these processes (Schuetz et al., 1998; Kretschmer and Baldwin, 2005). Modulation of the receptors levels could represent another mechanism of effect of PCBs on retinoid signaling occurring in complex mixtures of chemicals, some of which are probably still unknown (Schwarzenbach et al., 2006).

PCBs and detoxification metabolism enzymes: CYPs and NRF2

Human and animals are exposed to potentially toxic chemicals from both endogenous and foreign sources. To counter toxic insults and to maintain the homeostatic balance in important metabolic pathways, defense systems have been developed comprising enzymes and transport proteins capable of biotransformation reactions and subsequent elimination of endobiotics and xenobiotic metabolites. As described above, CAR , PXR and AhR function as sensors of toxic by-products, of endogenous metabolic compounds and of exogenous chemicals is facilitate their elimination. (Moore et al., 2003; Timsit and Negishi, 2007).

Recent studies have shown the existence of cross-talk between xenosensors and other NRs or transcription factors controlling endogenous signaling pathways including lipid metabolism and maintenance of glucose homeostasis (Moore et al., 2003; Lim and Huang, 2008). Such cross-talks could be at the root of alteration of physiological functions by xenobiotics and drugs provoking, among others, endocrine disruptions.

Although most of the biotransformation reactions occur in the liver, xenosensor-dependent multi-enzymatic complexes are also expressed in gastrointestinal tract, lung, kidney, brain, and placenta (Lamba et al., 2004; Weier et al., 2008).

Major reactions in phase I include oxidation (e.g., hydroxylation and deamination), reduction (e.g., addition of hydrogen atoms), and hydrolysis (e.g., splitting of ester and amide bonds). One of the most important characteristics of the phase I reactions is that a toxicant may acquire a functional group, such as –OH, –NH2, –COOH, or –SH, to form a product called primary metabolite. Members of the CYP super-family are expressed within the liver, intestine and kidney, the primary organs for uptake, metabolism, and excretion of xenobiotics (Michalets, 1998; Yu, 2001; Kliewer et al., 2002). The CYP microsomal enzymes represent a supergene family of heme proteins that are involved selectively in the metabolism of endogenous chemicals and xenobiotics (Plant, 2007). Fifty-seven CYP genes are present in humans, members of families CYP1, CYP2, CYP3, CYP4, and CYP7 playing crucial roles in xenobiotic metabolism (Nakata et al., 2006). The CYP3A sub-family represents the most relevant group. Indeed, CYP3A enzymes are the most abundant CYPs in human liver, comprising 30–50% of CYPs, and hence representing the bulk of the CYP enzymes that a chemical is likely to be exposed to (Cholerton et al., 1992).

Contrary to the CYPs, mainly localized in the smooth endoplasmic reticulum, phase II enzymes are located in the cytoplasmic matrix (Yu, 2001). The phase II metabolizing or conjugating enzymes belong to many super-families including glutathione-S-transferase (GST). Increased

hydrophilicity of xenobiotics, obtained by conjugation reactions catalyzed by phase II enzymes, generally enhances their excretion in the bile and/or urine. However, xenobiotic conjugation by phase II enzymes could result in the biosynthesis of toxic metabolites.
An important feature for regulation of phases I and II enzymes, as well as of phase III transporters, is that they may be induced to higher levels of expression following exposure to specific substrates, as well as to structurally unrelated compounds (Yu, 2001) because in some cases they are target of NRs induction. Indeed CAR coordinates the regulation of multiple hepatic genes resulting, in most cases, in metabolic detoxification by CYPs and transferases within the hepatocyte. For example, CAR prevents the induction of CYP4A, that, together with Superoxide Dismutase 3 (SOD3) may suppress oxidative stress (Swales and Negishi, 2004). PXR serves as a master transcriptional regulator of CYP3A isozymes (Quattrochi and Guzelian, 2001). Besides regulating members of the CYP families, PXR is involved in other aspects of xenobiotic metabolism, regulating GST, UGT, SULT, several MRPs, and OATP2 (Maglich et al., 2002).

CYP1A1

CYP1A1 contributes to the toxicity of many carcinogens, especially PAHs (Ma, 2001). The main pathway for CYP1A1 induction is through activation of the AhR pathway (Whitlock, 1999). AhR activators, as TCDD considered the most potent inducer, highly induce CYP1A1 mRNA levels and enzyme activities. Remarkably also the PXR/CAR activators induce CYP1A1 transcript levels, although to much lower levels than real AhR agonists. (Westerink et al., 2007).
PCB153 significantly enhanced the activity of CYP1A1 and the formation of micronuclei, but reduced the activity of GST (Wei et al., 2009). CYP1A1 and/or CYP1A2 induction by PXR/CAR activators has been observed in other studies as well (Gross-Steinmeyer et al., 2005).

CYP1A2

CYP1A2 represent 10-15% of total hepatic CYPs enzymes. CYP1A2 activates PAHs, nitrosamines and aryl amines into DNA-binding forms (Hecht, 1998). CYP1A2 induction on transcriptional level can occur through AhR dependent and independent pathways (Hung et al., 2012) or through post-transcriptional pathways (Silver et al., 1990). AhR activators

induced the CYP1A2 transcript levels. (Westerink et al., 2007). Recent findings suggest that at high concentrations, some AhR agonists trigger a cross-talk with the CAR/PXR pathway (Coe et al., 2006).
Moreover studies on humans who were exposed to high toxic levels of DL chemicals (PCDFs and PCBs), reported marked induction of CYP1A2 activity and this induction was an excellent biomarker of the exposure and adverse human health effects (Hung et al., 2012).

CYP3A4

The CYP3A represent approximately 30% of all CYP450 isozymes present in the liver and being characterized by a broad substrate specificity, CYP3A4 metabolizes approximately 50% of the drugs that are currently on the market and plays an important role in the detoxification of compounds (Vignati et al., 2005). CYP3A4 is regulated by CAR and PXR receptor (Moore et al., 2003). Cross-talk of the AhR agonists with the PXR/CAR pathways lead to induction of CYP3A4. (Faucette et al., 2006).

CYP2C9

The CYP2C subfamily contains four members of which CYP2C8, CYP2C9, and CYP2C19 are expressed in the liver (Goldstein and de Morais, 1994) where CYP2C9 represents 18% of total hepatic CYPs enzymes. Accumulating evidence indicates that CYPC9 ranks second, after CYP3A4, among the most expressed drug-metabolizing enzymes in human liver (Miners and Birkett, 1998). Little is known on the inducibility of this gene in response to xenobiotics in humans. The concentration and time dependence of CYP2C9 mRNA expression in response to inducers were consistent with the possible implication of at least three receptors GR, PXR, CAR (Gerbal-Chaloin et al., 2001; 2002) that bind two functional responsive elements in the regulatory region of gene CYP2C9. The presence of these two elements provides the mechanistic basis for the induction of CYP2C9 by dexamethasone and phenobarbital in primary human hepatocytes.

NF-E2-related factor 2 (Nrf2)

The transcription factor NF-E2-related factor 2 (NRF2) was demonstrated to regulate the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes. Several studies demonstrated that NRF2 is a key factor for cytoprotection in various aspects, such as anticarcinogenicity, neuroprotection and antinflammatory response determined by the coordinated actions of various categories of target genes. The activation mechanism of NRF2 implyies that, under normal conditions, NRF2 localizes in the cytoplasm where it interacts with the actin binding protein, Kelch-like ECH associating protein 1 (Keap1), being rapidly degraded by the ubiquitin-proteasome pathway. Signals from reactive oxygen species or electrophilic insults target the NRF2-Keap1 complex, dissociating NRF2 from Keap1. Stabilized NRF2 then translocates to the nuclei and transactivates its target genes.
NRF2 plays a critical role in genes expression regulation of enzymes family of glutathione transferase (GST), NAD(P)H quinone oxidoreductase and other phase II enzymes as UDP1A6, aldehyde reductase and antioxidant protein as superoxide dismutase (SOD), catalase, glutathione reductase (Kobayashi et al., 2005)and genes involved in glutathione biosynthesis (Moll et al., 2005). Recent evidence suggests that cross talk may exist between AhR and NRF2 pathways (Shin et al., 2007).

PCBs and oxidative stress biomarkers

Oxidative stress is associated with increased production of oxidizing species, including free radicals and peroxides, or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione. Oxidative stress is suspected to be play a role in neurodegenerative (Patel and Chu, 2011), and cardiovascular diseases by causing direct damage to the DNA (Evans and Cooke, 2004).
It is possible that the carcinogenic effects of TCDD may, in part, reside in the capacity of the induced CYP enzymes to leak oxidants and thus promote cell division (Cerutti et al., 1991) and oxidative DNA damage (Park et al., 1996).
Evidence suggests that the oxidative stress induced by contaminants such as PCB 77, TCDD is due to the interaction of these compounds with the AhR and activation of the CYP1A subfamily. Induction of CYP1A1 or CYP1A2 may lead to generation of excess levels of reactive oxygen species (ROS) (Schlezinger and Stegeman, 2001), resulting in cell injury (Hennig et al., 2001).

The CYP catalytic cycle involves binding of the substrate followed by binding and activation of molecular oxygen to a reactive intermediate, which hydroxylates the substrate. The formation of hydrogen peroxide and water as end products of CYP catalysis has been well described. A postulated mechanism of production of activated O_2 is the autoxidation of the oxycytochrome P450 complex, generating super- oxide (Kuthan and Ullrich, 1982; Ingelman-Sundberg and Johansson, 1984). The superoxide anion can, in turn, spontaneously dismutate, generating hydrogen peroxide as a byproduct. If transition metals are present to catalyze the one-electron reduction of hydrogen peroxide, hydroxyl radicals will be produced, leading to the indiscriminant damage to cellular biomolecules (Park et al., 1996).
However, superoxide anions, hydrogen peroxide, and hydroxyl radicals can also be generated during fatty acid metabolism which can modulate the effect of metabolites that are being formed. CYP2C9 is reported to be a significant source of ROS in coronary arteries (Fleming et al., 2001).
Moreover Hennig et al. (2002) have shown that PCB77 (an AhR ligand agonist) significantly disrupts endothelial barrier function, while PCB153 (not an AhR ligand but a CAR activator) does not (Toborek et al., 1995). In addition, PCB77, but not PCB153, contributes markedly to cellular oxidative stress, manifested by increased activity and content of CYP1A.
PXR activation is a risk factor for oxidative stress caused by an imbalance between the production of reactive oxygen species and detoxification of the reactive intermediates (Gong et al., 2006).

Reactive Oxygen Species (ROS)

Oxygen is an essential component of living organisms. The generation of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen is inevitable in aerobic metabolism of the body and can be formed by prooxidative enzyme systems, lipid oxidation, irradiation, inflammation, smoking, air pollutants, and glycoxidation (Halliwell 1997; Stief 2003). Reactive oxygen species cause lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression. In the past several years, unprecedented progress has been made in the recognition and understanding of roles of reactive oxygen species in many diseases (Cohen et al., 2000; Packer and Weber 2001). The body protects itself from the potential damages of ROS. Its first line of defense is SOD, glutathione peroxidases (GPx) and catalase (Lee, 2004).

Free radicals are generally unstable, highly reactive, and energized molecules. ROS also have been known to induce apoptosis of cells. Benign functions of free radicals have been reported, including the activation of nuclear transcription factors, gene expression, and a defense mechanism to target tumor cells (Simon et al., 2000).
Previous studies have shown that exposure to contaminants may cause oxidative stress. This can occur, for example, through activation of the CYP450 enzyme system which can lead to excess ROS generation during biotransformation processes.

GSH-GSSG Ratio

There are many redox couples in a cell that work together to maintain the redox environment; the GSH/GSSG couple is the most abundant redox couple in a cell. Changes of the half-cell reduction potential (Ehc) of the GSH/GSSG couple appear to correlate with the biological status of the cell (Schafer and Buettner, 2001). A dramatic change in the GSSG/GSH ratio indicates that redox metabolism is altered and shifts in biological ratios of redox couples are the consequences of enzymatic reactions due to GST and GPx activity (Flohé, 2012).
Indications of contaminant influence on glutathione status were also discovered. A high GSH/GSSG ratio is generally considered an index of healthy antioxidant defense, because more of the total glutathione is in a reduced state (Halliwell and Gutteridge, 2007). However, if the total concentration of glutathione decreases, a shift in the GSH/GSSG ratio towards an elevated GSH may occur as a compensatory mechanism to maintain the required level of antioxidant function. Hegseth et al., 2011 suggested that high halogenated organic compounds (HOC) concentrations can alter the glutathione ratio. Further investigations revealed a nonlinear, polynomial relationship between glutathione levels and PCB concentrations. This model suggests that HOC exposure induced a biphasic response with a counteractive, or compensatory, increase in glutathione levels up to a certain ΣPCB concentration. This was then followed by a toxic response, observed as a dose-dependent decrease in glutathione levels with higher PCB concentrations. Similar results have been obtained in previous studies using other classes of pollutants (Hegseth, 2011).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) catalyzes the destruction of the O_2- free radical into oxygen and hydrogen peroxide. It protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals (Fridovich, 1995). In humans three forms of superoxide dismutase are present. There are two main forms of SOD in cells. One form, found primarily in the cytoplasm, contains Cu and Zn (Cu,ZnSOD). The other form, found predominantly in mitochondria, contains Mn (MnSOD; Afonso et al., 2007). SOD activities in various diseases appear to be of clinical interest. The specific activity of Cu,ZnSOD is increased in in serum of patients with liver diseases.
ROS-initiated oxidative stress can be regulated by cell defense mechanisms, which include enzymatic antioxidants such as SOD and GPx (Halliwell and Gutteridge, 2007).

Adipose tissue as PCBs target organ

For decades, adipose tissue was considered an inert mass of stored energy with some advantageous properties, such as its function as an insulating substance and as a mechanical support for more important structures. However, the past fifteen years has seen a surge of interest in the study of adipose tissue, from its physiology to its developmental biology.
Adipocytes are essential regulators of whole-body energy homeostasis. These cells secrete several proteins that regulate processes as diverse as haemostasis, blood pressure, immune function, angiogenesis and energy balance (Lau et al, 2005). Although many cell types contain esterified lipids, adipocytes are unique in the quantity of lipid that they can store, the rapid release of these calories for use by other organs, and their secreted-protein repertoire. Adipocytes are found in stereotypical depots throughout the body, but can also be found mixed with other cell types in other locations, especially in loose connective tissue. There are two types of adipocyte, brown and white, which differ in several important properties. Even among white adipocytes, cells from different locations can have distinct molecular and physiological properties (Gesta et al, 2006). For example, increased visceral adipose tissue is associated with an increased risk of insulin resistance and cardiovascular disease, whereas increased subcutaneous adipose tissue is not. Adipocytes in visceral depots are sensitive to lipolytic stimuli, whereas adipocytes from structural depots (such as around the eyes and in the heel pads) do not release stored lipid easily. Most of our knowledge about adipogenesis comes from *in vitro*

studies of fibroblasts or pre-adipocytes. Adipocytes derive from multipotent mesenchymal stem cells.

The growing concern on adipocytes role is also related to the global increasing incidence of obesity with the consequent increase in associated morbidity and mortality (Giorgino et al, 2005).

The prevalence of obesity has increased at an alarming rate, with 65.4% of the adult population in the United States overweight and 30.5% of adults exhibiting obesity (Flegal et al. 2002; Hedley et al. 2004). Even more alarming, the prevalence of overweight children in the age range of 6–11 years increased from 4.2% in 1963 to 15.3% in 1999–2000 (Ogden et al. 2002). Reaven (1988) noted that several risk factors for cardiovascular disease cluster around an obesity phenotype, termed the metabolic syndrome. Some authors tried to define mechanisms contributing to the development of obesity and that link obesity to cardiovascular disease.

Interesting, recent findings suggest that in non diabetics with the metabolic syndrome, PCBs were linearly associated with waist circumference (Lee et al. 2007). Because serum concentrations of PCBs show decreasing trends but obesity is at epidemic proportions, the authors suggested that the toxicity of PCBs may synergistically increase as people become obese (Lee et al. 2007). Despite the potential for adipocytes to be frequently exposed to PCBs, the specific effects of PCBs on adipocyte function have not been defined. Moreover, the impact of enhanced PCB sequestration in the expanded adipose mass with obesity on the development of obesity-associated cardiovascular diseases is unknown (Arsenescu et al, 2008).

Low concentrations of PCB77 or TCDD increased adipocyte differentiation, glycerol-3- phosphate dehydrogenase activity, and expression of PPARγ, whereas higher concentrations inhibited adipocyte differentiation. PCB-77 promoted the expression and release of various proinflammatory cytokines from 3T3-L1 mouse adipocytes. Arsenescu et al, (2008) findings suggest that PCB77 may contribute to the development of obesity and obesity-associated atherosclerosis.

The murine 3T3-L1 or human SGBS cells undergo a pre-adipose to adipose like conversion as they progress from a rapidly dividing to a confluent and contact inhibited state. Although there have been attempts to describe distinct cellular intermediates between these cells and mature adipocytes, such intermediates have been difficult to characterize at the molecular level, and for practical purposes most workers in the field describe two phases of adipogenesis. The first phase, known as determination, involves the commitment of a pluripotent stem cell to the adipocyte lineage. Determination results in the conversion of the stem cell to a pre-adipocyte,

which cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types. In the second phase, which is known as terminal differentiation, the pre-adipocyte takes on the characteristics of the mature adipocyte. It acquires the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins. The molecular regulation of terminal differentiation is more extensively characterized than determination because most studies have used cell lines that have a restricted potential to differentiate into other cell types (Rosen and MacDougald, 2006).

Some pre-adipocyte models (for example, the mouse cell lines 3T3-L1, 3T3-F442A) undergo one or two rounds of cell division prior to differentiation, whereas others (such as mouse C3H10T1/2 and human pre-adipocytes) differentiate without post-confluence mitosis. Whether 'mitotic clonal expansion' is required for differentiation is controversial (Otto et al, 2005); however, it is clear that some of the checkpoint proteins for mitosis also regulate aspects of adipogenesis.

Adipocyte differentiation involves a temporally regulated set of gene-expression events and understanding the underlying transcriptional networks is of fundamental importance (Rosen and MacDougald, 2006).

Over the past two decades, attention has centered on the role of the nuclear receptor PPARγ and members of the C/EBP family in adipogenesis (Rosen et al, 2000).

Peroxisome Proliferator-Activated Receptor γ (PPARγ)

As described before, PPARγ is a member of the NRs superfamily and is considered "the master regulator of adipogenesis" (Rosen et al, 2000). Forced expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts (Tontonoz and Spiegelman, 1994), and no factor has been discovered that promotes adipogenesis in the absence of PPARγ. These findings are consistent with the observation that most pro-adipogenic factors seem to function at least in part by activating PPARγ expression or activity. Indeed, the pro-adipogenic C/EBPs and Krüppel-like factors (KLFs) have all been shown to bind at least one of the two PPARγ promoters. Efforts to identify an endogenous PPARγ ligand have been largely unsuccessful. Cyclic AMP (cAMP)-dependent ligand activity was found in 3T3-L1 cells in the first two days of differentiation, after which this activity rapidly declined (Tzameli et al., 2004). This surprising finding

indicates that ligand activation of PPARγ is required to induce adipogenesis but not to maintain PPARγ-dependent gene expression in mature adipocytes. Other studies have found that the transcription factors sterol response element-binding protein-1c (SREBP1c) and C/EBPβ can increase PPARγ ligand production (Hamm et al., 2001; Kim et al., 1998), but these findings have not led to the identification of a definitive endogenous PPARγ agonist. PPARγ is not only crucial for adipogenesis but is also required for maintenance of the differentiated state.
It has been demonstrated that low concentrations of PCB77 or TCDD increase adipocyte differentiation and expression of PPARγ, whereas higher concentrations inhibit adipocyte differentiation suggesting that PCB-77 may contribute to the development of obesity (Arsenescu et al., 2008).

Genes involved in adipogenesis upstream Pparγ induction

Hairy and enhancer of split-1 (HES1)

The process of adipogenesis involves a complex program of gene expression that includes down-regulation of the gene encoding Hes-1, a target of the Notch signaling pathway. To determine if Notch signaling affects adipogenesis, 3T3-L1 were exposed preadipocytes to the Notch ligand Jagged1 and found that differentiation was significantly reduced.
The Notch proteins are cell surface receptors activated by the Delta and Jagged/Serrate families of ligands. Interaction with a ligand leads to two proteolytic cleavage events that release the Notch intracellular domain (NICD) from the plasma membrane. NICD translocates into the nucleus, where it interacts with the DNA binding protein CSL (CBF-1, Suppressor of Hairless, LAG-1, RBP-J; Aster et al., 1997). Several genes that are directly activated by the NICD-CSL complex have been identified. The best characterized of these are the HES and HRT families of genes, all of which encode transcriptional repressors (Nakagawa et al., 1999). Hes-1 is a basic helix-loop-helix (bHLH) DNA binding protein related to Drosophila Hairy and Enhancer of Split proteins and forms homodimers as well as heterodimers with other HLH proteins.
Notch signaling is generally thought to control cell fate decisions by inhibiting the development of certain lineages and/or promoting the development of others.

Hes-1 was identified among a large group of genes whose expression is down-regulated during adipogenesis *in vitro* and *in vivo* (Soukas et al., 2001). Although Hes1 expression can be stimulated by growth factors, its connection to the Notch pathway prompted us to examine the relationship between Notch signaling and adipogenesis. We found that Notch signaling through Hes-1 can profoundly inhibit the differentiation of 3T3-L1 preadipocytes. Interestingly, artificially reducing Hes1 expression also inhibited adipogenesis, and this correlated with the induction of the adipogenic inhibitor DLK/Pref-1. Ross (2004) suggests that Hes-1 has two roles in adipogenesis: one promotes adipogenesis, possibly through the down-regulation of inhibitory proteins such as DLK/Pref-1, and the other inhibits adipogenesis at a step prior to the induction of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ).

Protein delta homolog 1 (Dlk1)

This gene encodes a transmembrane protein containing six epidermal growth factor repeats. The protein is involved in the differentiation of several cell types, including adipocytes; it is also thought to be a tumor suppressor. This gene is expressed from the paternal allele. A polymorphism within this gene has been associated with child and adolescent obesity. The mode of inheritance for this polymorphism is polar overdominance; this non-Mendelian inheritance pattern was first described in sheep with the callipyge phenotype, which is characterized by muscle hypertrophy and decreased fat mass (provided by RefSeq). DLK1 levels play an important roles in growth signaling networks. DLK expression is up-regulated in 3T3-L1 cells induced to differentiate into adipocytes. The PPARγ agonist rosiglitazone was found to increase DLK expression in 3T3-L1 cells, indeed PPARγ is required for the transcriptional activation of the DLK gene (Couture and Blouin, 2011).

CCAAT-enhancer-binding proteins (C/EBPs)

Several C/EBP family members, including C/EBPα, β, γ and δ and Ddit3 (DNA damage-inducible transcript 3), are expressed in adipocytes. The temporal expression of these factors during adipocyte differentiation indicates a cascade whereby early induction of C/EBPβ and C/EBPδ leads to induction of C/EBPα. This notion is further supported by sequential binding of these transcription factors to several adipocyte promoters during

differentiation. C/EBPβ is crucial for adipogenesis in immortalized preadipocyte lines, but its effect is less obvious in embryonic fibroblasts. C/EBPβ and C/EBPδ promote adipogenesis at least in part by inducing C/EBPα and PPARγ expression. C/EBPα induces many adipocyte genes directly, and *in vivo* studies indicate an important role for this factor in the development of adipose tissue (Linhart et al., 2001). Despite the importance of C/EBPs in adipogenesis, these transcription factors clearly can not function efficiently in the absence of PPARγ (Rosen et al., 2002). Expression of exogenous PPARγ in C/EBPα-deficient cells showed that, although C/EBPα is not required for accumulation of lipid and the expression of many adipocyte genes, it is necessary for the acquisition of insulin sensitivity (Wu et al., 1999; El-Jack et al., 1999).
Ddit3 encodes a member of the C/EBP family of transcription factors. The protein functions as a dominant-negative inhibitor by forming heterodimers with other C/EBP members, such as C/EBP and LAP (liver activator protein), and preventing their DNA binding activity. The protein is implicated in adipogenesis and erythropoiesis, is activated by endoplasmic reticulum stress, and promotes apoptosis (provided by RefSeq).
Moreover other important genes involved in differentiation and metabolism of preadipocytes, are target of endocrine disruption.

Nuclear receptor co-repressor 2 (Ncor2)

The NCoRω splice variant inhibits, whereas the NCoRδ splice variant promotes, adipogenesis. Furthermore, the ratio of NCoRω to NCoRδ decreases during adipogenic differentiation. This alteration in corepressor splicing helps convert the cellular transcriptional program from one that maintains the pre-adipocyte in an undifferentiated state to a new transcriptional context that promotes differentiation and helps establish the proper physiology of the mature adipocyte (Goodson et al., 2011).
This gene encodes a nuclear receptor co-repressor that mediates transcriptional silencing of certain target genes. The encoded protein is a member of a family of TH- and RAR-associated co-repressors. This protein acts as part of a multisubunit complex which includes histone deacetylases to modify chromatin structure that prevents basal transcriptional activity of target genes. Co-repressors, such as NCoR show anti-adipogenic effect decreasing PPARγ activity and reducing 3T3-L1 adipogenesis; their reduction promotes differentiation (Yu et al., 2005).

Genes involved in adipogenesis downstream Pparγ induction

Cyclin-dependent kinase inhibitor 1B (Cdkn1B)

CDKIs may regulate establishment of adipocyte number *in vivo* suggested by marked changes in p27 and p21 during differentiation in 3T3-L1 preadipocytes.
This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and inhibit the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes involved in the cell cycle progression at G1. CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes triggered the degradation of this protein, required for the cellular transition from quiescence to the proliferative state (provided by RefSeq).
Adipocyte hyperplasia is characteristic of some forms of human obesity, but the role of adipocyte number in obesity and how normal adipocyte number is established are unclear. Preadipocytes proliferate and then differentiate to become mitotically quiescent adipocytes. This involves exit from the cell cycle, a process regulated by cell cycle inhibitors such as the cyclin-dependent kinase inhibitors (CDKIs) p27 and p21. 3T3-L1 preadipocytes show marked changes in p27 and p21 during differentiation, suggesting CDKIs may regulate establishment of adipocyte number *in vivo* (Naaz et al., 2004).

Sonic hedgehog (Shh)

The hedgehog (Hh) signaling pathway is crucial in the development of all known animals. In the embryo, it regulates morphogenesis of a variety of tissues and organs, in the adult, it controls stem cell proliferation. Members of these family are expressed at different times of development and in specific cell types and they are tightly controlled by highly complex, yet divergent transcriptional enhancers. This gene encodes a protein that is instrumental in patterning the early embryo. Addition SHH or its activated receptor inhibits differentiation in 3T3-L1 pre-adipocytes and C3H10T1/2 cells (Zehentner et al., 2000; Suh et al., 2006). Studies involved adipose-derived stromal cells (ASCs) capable of differentiating into osteogenic and adipogenic cell types showed that Hedgehog signaling increased during early osteogenic differentiation (Shh, Ptc1, and Gli1), but decreased during adipogenic differentiation. Conversely, blockade of endogenous Hedgehog

signaling, with the Hedgehog antagonist cyclopamine, enhanced adipogenesis at the expense of osteogenesis (James et al., 2010).

Tafazzin (Taz)

TAZ was expressed in postconfluent 3T3-L1 preadipocytes and downregulated during differentiation. Downregulation of TAZ was specifically mediated by dexamethasone (DEX), one component of induction cocktails routinely used in adipocyte differentiation. (He et al., 2012). TAZ activity suppresses PPARγ-stimulated adipocyte differentiation (Byun et al., 2012) and suppresses adipocyte development by interacting with PPARγ (Jang et al., 2012).
The proteins encoded by these genes are leucine zipper proteins, that function as transcriptional regulators. The expression of this gene is stimulated by glucocorticoids and interleukin 10, and it appears to play a key role in the anti-inflammatory and immunosuppressive effects of this steroid and chemokine (provided by RefSeq).

Vitamin D receptor (VDR)

Emerging data suggest a role for the vitamin D receptor (VDR) in lipogenesis and adipocyte differentiation (Wood, 2008).
Vitamin D receptors (VDRs) are members of the NR1I family and form heterodimers with members of the RXR family. VDR is expressed in the intestine, thyroid and kidney and has a vital role in calcium homeostasis.
VDR level changed during differentiation of pre-adipocytes into adipocytes. Overall, recent observations make a strong case for the role of vitamin D in adipogenesis mediated through VDR-dependent inhibition of C/EBPα and PPARγ expression in a dose-dependent manner by 1,25-dihydroxyvitamin D (Kong and Li, 2006) and a decrease in PPARγ transactivation activity partially inhibiting endogenous PPARγ ligand formation (Blumberg et al., 2006).

AIMS

The aims of the project are:

- to highlight the effects of 21 PCB congeners grouped in three mixtures on *in vitro* models representing two PCB toxicological targets: liver, involved in metabolic process, and adipose tissue, representing the PCB storage;
- to characterize a panel of biomarkers of effects related to PCB exposure;
- to evaluate the effects PCB mixture effects in order to characterize specific mechanisms of action.

The 21 congeners have been grouped into three mixtures one including DL-PCBs and two featuring NDL-PCBs. The NDL-PCB congeners have been further grouped into two mixtures on the basis of structural and modes of action similarities according to the classification proposed by Wolff et al. (1997) and Negri et al. (2003), namely Mix 1, the estrogenic and variably persistent congeners (PCB 44, 49, 52, 101, 174, 177, 187, 201), with one or two unsubstituted para positions and Mix3, highly persistent in the organism and phenobarbital-like inducers of CYP1A1, 2B and 2A1 (PCB 99, 153, 180, 183, 196, 203). Mix 2 includes non-ortho and mono-ortho congeners with dioxin-like properties AhR-mediated, potentially anti-estrogenic and CYP1A and 2A1 enzyme-inducing (PCB 77, 81, 105, 114, 118, 126, 169).

The concentration of each congener into the mixtures has been derived from previous analysis on human adipose tissues analyzed in Italian general population (La Rocca et al., 2008) . In this work the PCB concentrations in fat has been transformed in blood circulating levels on the basis of cholesterol and triglycerides serum content, estimated in 4 mg/ml as maximum level in normal physiological conditions. As a consequence, the *in vitro* model is able to simulate the actual human exposure conditions in terms of mixture and levels of PCB.

Moreover the mixtures exposure allows to characterize specific mechanisms of action and effects as a contribute for the development of toxicological criteria for PCB risk evaluation.

Liver is highly responsive to endocrine regulation since it exhibits a high expression of nuclear receptors (NRs) and responsible for the PCBs detoxification metabolism through expression of enzymes involved in phase I, II and phase III xenobiotics detoxification. PCBs display interaction with a wide range of NRs shown to be target for EDs and selected as panel of possible early biomarkers of effect: Estrogen Receptors (ERα, ERβ), Aryl

Hydrocarbon Receptor (AhR), Costitutive Androstane Receptor (CAR), Pregnane X Receptor (PXR) involved in detoxifying phase I and II metabolic pathways. Moreover, PCBs interfere with Testosterone-Androgen Receptor (AR) binding, Peroxisome proliferator-activated receptor γ (PPARγ), retinoic acid receptor α (RARα) and Thyroid Hormone Receptor α (THRα) signaling and interact with NRF2, a transcription factor implied in the regulation of different antioxidative genes. Indeed phase I enzymes activity is responsible for reactive oxygen species (ROS) activating enzymes involved in cellular redox homeostasis.

Two hepatic cell lines have been used to assess different types of age-related vulnerability and to improve characterization of the effects of PCBs mixtures: a human hepatoblastoma cell line (HuH6), selected as in vitro model representative of liver of an infant and a human hepatocarcinoma cell line (HepG2) representative of a 15 years old male. A panel of selected NRs (ERα, ERβ, AR, AhR, CAR, PXR, PPARγ, RARα, THRα, NRF2), P450 enzymes (CYP1A1, 1A2, 2C9 and 3A4) and stress oxidative markers GSH-GSSG ratio, GST and SOD activity, ROS level) have been used as early biomarkers of PCB effects on *in vitro* models. Moreover gene expression profile has been evaluated in order to highlight different functional processes and pathways affected by the three PCB mixtures treatment performing Microarray assay on HepG2 cells, an *in vitro* model widely and deeply studied in the last decades. The slow metabolism of PCBs leads to bioaccumulation and storage in adipose tissues. Adipose tissue is a highly active metabolic and endocrine organ. The endocrine functions of adipose tissue involved the secretion of adipokines, leptin, adiponectin, resistin, TNFα, interleukin-6 and the expression of enzymes involved in the metabolism of steroid hormones (Kershaw and Flier, 2004). These important endocrine function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess and deficiency.

PCBs bioaccumulation and permanence in animal fat could affect preadipocyte programming and differentiation and adipocytes metabolism through mis-regulation of critical pathways involved in adipogenesis, lipid metabolism, or energy balance (Grun and Blumberg, 2007). Previous results demonstrated that coplanar PCB77, an AhR ligand, promoted inflammation in endothelial cells (Lim et al., 2007) so inducing expression of inflammatory cytokines in adipocytes (Arsenescu et al., 2008). Induction of proinflammatory cytokines by PCBs in adipocytes would be anticipated to promote the development of obesity and obesity-associated cardiovascular disease (Mullerova and Kopecky, 2006).

Thererfore, the present study examines the effects of PCBs on adipose tissue in vitro model using two human and murine preadipocytes cell lines . Differentiation of 3T3-L1, the Mouse Embryonic Fibroblasts cell line, and SGBS, the human Simpson-Golabi-Behmel syndrome preadipocyte cell strain, was evaluated to determine the effect of PCBs exposure, evidencing species-specific differences in adipogenesis and response to PCB mixtures exposure. Moreover, gene expression of biomarkers involved in adipocytes differentiation and metabolism (Hes1, Lrp5, Ncor2, Shh, Taz, Tsc22D3, Vdr, Dlk1, Cdkn1B, Ddit3) were evaluated by qPCR in 3T3-L1. The part of the study regarding preadipocytes differentiation were performed in the laboratory of prof. Timo Hamers in the Institute for Environmental Studies (IVM), VU University Amsterdam.

MATERIALS AND METHODS

Chemicals

PCB: standard solutions and mixtures composition

PCB 44, 49, 52, 99, 101, 105, 114, 118, 153, 180, 183, 187, 196 and 201 were purchased from Chem Service (West Chester, PA, USA); PCB174, 177 and 203 from Ultra Scientific (N. Kingstown, RI, USA); PCB 77, 81, 126, 169 from Dr. Ehrenstorfer (Augsburg, Germany). All PCB standards were purchased as iso-octane solutions.

In previous works, PCB congeners concentrations were determined in human adipose tissues of an Italian population (La Rocca et al, 2008; Turrio Baldassarri et al., 2002). In order to adapt such concentrations, expressed in pg/mg fat, to blood circulating levels, we considered the fat serum content as the sum of cholesterol and triglycerides. This is estimated in 4 mg/ml as maximum level in normal physiological conditions, according to the National Institute of Health (NIH Publicaton, 2002) and the American Hearth Association (http://www.heart.org/HEARTORG). Each PCB congeners concentration in fat was then multiplied by 4 thus obtaining pg/ml values, conceivably representing PCB concentrations in human blood, in turn reflecting internal organ exposure (Tab. 1). Calculated concentrations of each PCB standard solutions were mixed together according to the proposed classification of Wolff et al. (1997) and Negri et al. (2003), based on structural and modes of action similarities, to obtain 3 different standard mixture solutions in n-hexane (Carlo Erba, Milan, Italy): MIX1 (one or two unsubstituited para positions) potentially estrogenic (PCB 44, 49, 52, 101, 174, 177, 187, 201); MIX2 (non-ortho and mono-ortho substituted) dioxin-like properties AhR-mediated, potentially anti-estrogenic, CYP1A and CYP2A1 enzyme-inducing (PCB 77, 81, 105, 114, 118, 126, 169); MIX3, highly persistent in the organism, phenobarbital-like inducers of CYP1A1, CYP2B and CYP2A1 (PCB 99, 153, 180, 183, 196, 203).

Before use, solvent was eliminated by gentle nitrogen flushing and PCB mixtures were dissolved in DMSO (Euro Clone, Milan, Italy) as concentrated solutions to be conveniently diluted in cell culture medium during cell exposure experiments. The composition of each mixture and final concentrations of each PCB congener in cell culture medium are shown in Tab 1:

Mix1			Mix2			Mix3		
congener	pg/ml	pM	congener	pg/ml	pM	congener	pg/ml	pM
44	0.759	2.599	77	0.407	1.394	99	41.848	128.198
49	0.602	2.062	81	0.226	0.774	153	288.947	800.077
52	1.280	4.384	105	14.776	45.265	180	202.199	511.478
101	2.233	6.841	114	3.383	10.364	183	29.570	74.800
174	1.184	2.995	118	68.309	209.259	196+203	22.887	55.5748
177	14.607	36.950	126	0.256	0.784			
187	53.201	134.576	169	0.132	0.366			
201	18.564	43.195						

Table 1- Concentrations of PCB congeners within each mixture, used for HuH6, HepG2 and 3T3 cell treatments. Such concentrations correspond to 1× dilution used in the cytotoxic assay and in cells exposure experiments. Values are expressed in pg/ml and in pM final concentrations in cell culture medium (Tait et al, 2011)

HuH6, HepG2 human hepatic cell lines: analysis of biomarkers of effect at gene expression and protein level

Hepatic cell lines and culture conditions

Human hepatoblastoma cell line (HuH6, established from hepatoblastoma of a 1-yr old male donor) was kindly obtained from Dr. Marco Salvatore (National Centre for Rare Diseases, Istituto Superiore di Sanità, Rome, Italy;). Human hepatocarcinoma cell line (HepG2) derived from the liver tissue of a 15 year old Caucasian American male, were kindly obtained from Dr. Anna Ruggieri (Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy). HuH6 and HepG2 were grown in Dulbecco Modified Eagle's medium F12 without phenol red (Technologies, Paisley, UK), supplemented with 10% fetal bovine serum (Lonza), 2 mM l-glutamine (Lonza), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Cells were maintained in a humidified Steri-Cult 200 Incubator (Forma Scientific, Marietta, OH, USA) at 37 °C and 5% CO_2.

HuH6 and HepG2 cell lines treatment

Culture flasks were plated with $1x10^6$ cells per flask in culture medium and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion.

Both cell lines were treated with each of the three mixtures or with medium alone as control at final concentrations as reported in Table 1, and incubated for 72h at 37 °C. Treatments were performed in triplicate to obtain three biological replica.
At the end of incubation, medium supernatants were collected and stored at -80 °C until use. Cell monolayers were trypsinized and harvested for further processing.

Cytotoxicity assay

Cytotoxicity was evaluated by the MTS assay (Cell Titer 96 Aqueous One Solution assay, Promega).
Briefly, 96 flat-bottomed multiwells were plated with 5,000 cells/well (one multiwell for each cell line) and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion. All treatments were performed in triplicate incubating the cells for 72h at 37 °C with each mixture diluted in culture medium. At the end, following 1h30min reaction with the MTS reagent at 37 °C, cell viability was determined by reading absorbance at 450 nm by a Victor 3 Multilabel Reader (PerkinElmer, Waltham, MA,USA).

Total RNA extraction and cDNA synthesis

HuH6 and HepG2 cell pellets were added with Qiazol Buffer (QIAGEN, Hilden, Germany) and stored at -80 °C until use.
Cell lysates were extracted for their total RNA content by using the RNAeasy Mini Kit (QIAGEN) according to manufacturer protocol. Total RNA quantity was determined by NanoDrop reading (NanoDrop, Wilmington, DE, USA) whereas integrity was evaluated by an 1% agarose gel electrophoresis.
All the samples were not degradated and with an absorbance ratio at 260/280 nm≈2.
An aliquot of total RNA (1µg) from each sample was retrotranscribed to cDNA using the cDNA Synthesis Kit (Quantace, London, UK) according to manufacturer protocol.

Gene expression evaluation by real-time PCR

Real-time PCR was performed in order to assess gene expression of the selected panel of target genes: ERα, ERβ, AR, PPARγ, RARα, AhR, CAR, PXR, THRα, THRβ, and Nrf2. Specific primers for nuclear receptors and

Nrf2 genes as well as for GAPDH, used as reference gene, were designed using the NCBI web application Primer - BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and purchased by Life Technologies (Life Technologies, Paisley, UK). Primer sequences are listed in Tab. 2. Lyophilized primers were dissolved in RNase/DNase-free water to obtain 100 mM stock solutions and then further diluted 1:10 to obtain 10 mM working solutions. Stock and working solutions were stored at -20 °C.

HS_ESR1_fw	ACTGCGGGCTCTACTTCATC
HS_ESR1_rev	GGCTGTTCCCAACAGAAGAC
HS_ESR2_fw	CTCTTTTGCCTGAAGCAACG
HS_ESR2_rev	CTGGGCAGTTAAGGAGACCA
HS_PPARγ_fw	GATGACAGCGACTTGGCAAT
HS_PPARγ_rev	AGGAGCGGGTGAAGACTCAT
HS_AhR_fw	TTCCACCTCAGTTGGCTTTG
HS_AhR_rev	GGACTCGGCACAATAAAGCA
HS_AR_fw	CCCATCTATTTCCACACCCA
HS_AR_rev	GCAAAGTCTGAAGGTGCCAT
HS_RARα_fw	GCTGAGAGGGCTTCCCCGGT
HS_RARα_rev	TCTCCTGGAGCCCCCACCCT
HS_THRα_fw	AGTCTCCGACGCCATCTTTGAACT
HS_THRα_rev	GTTGTGTTTGCGGTGGTTGACGTA
HS_CAR_fw	CGTCTTCCGTTCCCTGCCCAT
HS_CAR_rev	GGAGCAGGAGAGAAGAGGGCCA
HS_PXR_fw	CATACACCCCTTTGCTACGC
HS_PXR_rev	GAGTCTGTGGCTTCACTCCC
HS_GAPDH_fw	ACTCCTCCACCTTTGACGCT
HS_GAPDH_rev	CTTCAAGGGGTCTACATGGC
HS_NRF2_fw	CATTCACTGCATATCGATTGG
HS_NRF2_rv	GTAATCACTGACTTAAGTCA

Table 2: Primer sequences of NRs and NRF2 analyzed in liver cell lines

Experiments were performed in duplicate on 96 well PCR plates. Optimal conditions for real-time PCR reactions were set in terms of amplification efficiencies (95-100%) and primer concentrations using the standard curve method.

Gene	*Primer Concentration*
ESR1	800
ESR2	400
PPARγ	400
AhR	200
AR	200
RARα	800
THRα	800
CAR	800
PXR	200
GAPDH	400
Nrf2	400

Reactions were prepared using the SensiMix Plus SYBR kit (Quantace, London, UK) and were run on a Stratagene MX3005P instrument (Agilent Technologies, Santa Clara, USA).
The thermal program was as follows: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15s, 58 °C for 30s and 72 °C for 1 min; 1 dissociation cycle from 55 to 95 °C (30s/°C) to verify amplification products. Results were calculated as ΔΔct corrected for the amplification efficiencies according to the Pfaffl method (Pfaffl, 2001).

Cytochrome P-450 monooxygenases activity assays

CYP450 1A1, 1A2, 2C9, 3A4 enzymes activity was evaluated respectively by the P450-Glo™ CYP1A1 Assay, P450-Glo™ CYP1A2 Assay, P450-Glo™ CYP2C9 Assay, P450-Glo™ CYP3A4 Assay (Promega).
The CYP enzyme substrates are derivatives of beetle luciferin [(4S)-4,5-dihydro-2- (6´-hydroxy-2´-benzothiazolyl)-4-thiazolecarboxylic acid] which are converted by CYP enzymes to a luciferin product that is detected in a second reaction with the luciferin detection reagent. The amount of light produced in the second reaction is proportional to CYP activity. Cytochrome P450 substrate selectivity depends on the specific structure of the proluciferin substrate.
96 flat-bottomed multiwells were plated with 5,000 HuH6 cells/well or 15,000 HepG2 cells/well and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion. All treatments were performed in triplicate incubating the cells for 72h at 37 °C with each mixture diluted in culture medium at a final concentration as reported in Tab. 1. After 72h, treatment medium was removed washing treated cells with medium alone and then

incubating with medium containing the luminogenic CYP substrate as indicated in Tab. 3

Assay	Substrate	Final Substrate Concentration	Well Plate	Incubation Time
CYP3A4	Luciferin-IPA	3µM (1:1,000 dilution*)	60µl	30–60 minutes
CYP1A2	Luciferin-ME	50µM (1:50 dilution*)	60µl	3–4 hours
CYP2C9	Luciferin-H	100µM (1:50 dilution*)	60µl	3–4 hours
CYP1A1	Luciferin-CEE	100µM (1:50 dilution*)	60µl	3 hours

Table 3: Cytochrome P-450 monooxygenases activity assays protocol (Promega)

To determine background luminescence, luminogenic substrate were added in culture medium to a set of empty wells. Plates were incubated at 37°C for 30 minutes with Luciferin-IPA or 3 hours with other luminogenic substrates.
Following, an equal volume of Luciferin Detection Reagent was added to each well, that were mixed briefly on a multiwell plate shaker to form a lysate. 50µl of cell lysate were transferred from each well to a 96-well opaque white luminometer plate at room temperature, equilibrating plates at room temperature for 15 minute and reading luminescence with a Victor 3 Multilabel Reader (PerkinElmer, Waltham, MA,USA).

Superoxide Dismutase (SOD) Activity assay

SOD enzyme activity levels in each sample were evaluated by Superoxide Dismutase (SOD) Activity Assay Kit (Biovision, Mountain View, CA, USA).
Treated cells (see paragrapgh 2.2) were lysated in ice cold buffer 0.1M Tris/HCl, pH 7.4 containing 0.5% Triton X-100, 5mM 2-Mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA), 0.1mg/ml PMSF. Cell lysates were centrifuge at 14,000 x g for 5 minutes at 4°C. Cell debris were discarded whereas the supernatant, containing total SOD from cytosolic and mitochondria cell compartments, were collected and stored at -80 °C until use. Sample were added of 20 µl Sample Solution and plated in duplicate on 96 flat-bottomed multiwells. Each well were then added of 200 µl of WST

Working Solution and 20 µl of Enzyme Working solution. Control wells containing Kit solutions but no samples were also included. Moreover:

- Blank1 does not contain the Dilution buffer and contains water instead of the sample
- Blank2 does not contain neither water nor the Enzyme Working solution
- Blank3 does not contain the Enzyme Working solution and contains water instead of the sample

Plates were incubated at 37°C for 20 minutes. The absorbance was read at 450 nm using a Victor 3 Multilabel Reader (PerkinElmer).
SOD activity (inhibition rate%) were calculated using the following equation:

$$\text{SOD Activity (U/ml)} = \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \times 100$$

Oxygen reactive species (ROS) detection assay

The quantification of ROS was performed using the Reactive Oxygen Species (ROS) Detection Reagents (Life Technologies, Paisley, UK).
24 well flat-bottomed multiwells were plated with 30,000 HuH6 cells/well or 100,000 HepG2 cells/well and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion. Cells were treated with each PCB mixture in triplicate, at concentrations indicated in Table 1, for 72h at 37 °C.
After 72h treatment medium was removed to perform the experiment.
Shortly before performing the experiments, the ROS indicator was reconstituted using high quality anhydrous dimethylsulfoxide (DMSO) to make a concentrated stock solution 2mM (50µg CM-H_2DCFDA in 43µL DMSO).
Treated cells were washed with pre-warmed phosphate buffered saline (PBS) and incubated at 37°C for 30 minutes with PBS containing the ROS indicator to provide a final working concentration of 5 µM dye.
Following, cells were harvested using a cell scraper and centrifugated; supernatants were removed, cells were resuspended in 300 µl PBS and the fluorescence (F_{ex} 492–495 nm/F_{em} 517–527 nm) was read by a Victor 3 Multilabel Reader (PerkinElmer).

Reduced, oxidized, total glutathione (GSH) detection assay

The quantification of the reduced and total GSH was performed by using the ApoGSH^{TM} Glutathione Colorimetric Assay Kit (Biovision).
The assay is based on the glutathione recycling system by 5,5'-Dithiobis(2-nitrobenzoic) acid (DTNB) and glutathione reductase. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid which has yellow color. The oxidized GSH generated (GSSG) can be reduced back to GSH by glutathione reductase; in turn, GSH reacts with DTNB again to produce more 2-nitro-5-thiobenzoic acid. Therefore, the recycling system dramatically improves the sensitivity of total GSH detection. The kit can also specifically detect the reduced form of GSH by omitting the glutathione reductase from the reaction mixture. The sensitivity for detecting the reduced form of GSH (without recycling system) is 100 times lower than detecting the total GSH.
Treated and control cells (see 2.2 paragraph) were collect by centrifugation. Supernatants were removed and cell pellets were washed with 0.5 ml ice-cold PBS. Following centrifugation, cell pellets were lysed in 80 µl ice-cold GSH buffer and incubated on ice for 10 minutes.
20 µl of 5% 5-Sulfosalicylic acid (SSA) were added to each sample, centrifugating tubes to collect supernatants which contains GSH to be assayed.
Two standard curves were generated:

1. for detecting only the reduced form of GSH ,50, 40, 30, 20, 10, and 0 µl of the 1 µg/µl GSH standard were dispensed into microcentrifuge tubes adding 1% SSA solution to a final volume of 100 µl/tube.
2. for detecting the total GSH, 1 µg/µl GSH solution was diluted to 10 ng/µl with 1% SSA solution, dispensing 50, 40, 30, 20, 10, and 0 µl of such GSH standard solution into microcentrifuge tubes; then, 1% SSA solution was added to a final volume of 100 µl/tube.

Two parallel Reaction Mix was prepared for the standard and samples to be assayed in 96-well plate. One, to detect total GSH, contains the following reagents:20 µl NADPH Generating Mix,20 µl Glutathione Reductase,120 µl Glutathione Reaction Buffer
The other, to detect only reduced GSH, contain no Glutathione Reductase with same volume (20 µl) replaced by Glutathione Reaction Buffer.
160 µl of each reaction mix were added to each sample well, in duplicate for each determination, incubating at room temperature for 10 minutes to generate NADPH.

20 µl of either the GSH standard solutions or the sample solutions were added incubating the plate at room temperature for 8 minutes.
20 µl of substrate solution was added incubating the plate at room temperature for 5 minutes. The absorbance (OD) was read at 405 nm using Victor 3 Multilabel Reader (PerkinElmer). Calculation of Total GSH was performed subtracting OD of blank wells from samples OD. Reduced GSH and total GSH concentration were determined in each samples using respectively the reduced and total GSH calibration curves.

Gene expression profiling of HepG2 cell line

Technical procedure

cDNA and cRNA synthesis, amplification and labeling was performed using the Low Input Quick Amp Labeling Kit, Two Colors (Agilent, Santa Clara, CA, USA) with spike-in internal controls (Agilent) following manufacturer' s protocol. For each sample we performed two cDNA synthesis starting from two aliquots of 1 µg total RNA as input, consequently processed to obtain two differently labeled cRNA, with Cy3-CTP or Cy5-CTP, in order to perform two technical replica of array hybridizations (dye swap) per biological replica. As a whole, a total of twenty-four samples were obtained: six labeled samples per treatment (three with Cy3 per biological replica plus three with Cy5 per biological replica) and six labeled samples of the control samples (Cy3 and Cy5). Labeled samples were quantified with NanoDrop with a resulting yield>825ng of cRNA and a specific activity >8.0 pmol dye/µg cRNA, as requested by the protocol.
Labelled treated and control samples were co-hybridized (one labeled with Cy3 and one with Cy5, alternately) on 4×44 K Whole Human Genome Microarray Slides (Agilent) featuring ~41,000 unique human genes and transcripts. Hybridizations were carried out at 65 °C for 17h, with a 10 rpm rotation in an Agilent Hybridization Oven. After washing with Gene expression wash buffers (Agilent), slides were scanned by the Agilent G2505B Microarray Scanner System. Data extraction and quality control were performed through the Agilent Feature Extraction Software v9.5. All the arrays passed the quality control and were further analyzed.

Data analysis

Normalization and differential expression analysis were performed with the Limma package (Smyth et al., 2005) part of Bioconductor (Gentleman et al., 2004; www.bioconductor.org) within the R environment (R Development Core Team, 2009; http://www.R-project.org). Within-array normalization was applied on raw signal data of each array using the Global Loess Normalization method which contextually subtracts background to signal values and transform data in $\log_2$ Fold Change ($\log_2$ FC) values. An Aquantile between-array normalization was applied to fluorescence intensity values to guarantee a similar distribution across arrays.
Differential gene expression changes between treatments and controls were calculated applying a simple linear model fit and a moderated t-statistic for the significance analysis obtaining $\log_2$ FC averages and p-values for each gene and treatment. The application of a Benjamini-Hochberg correction for the false discovery rate (FDR) for multiple tests (Benjamini et al., 1995) generated an FDR-adjusted p-value for each gene across treatments. A threshold equal to 6 for $\log_2$ fluorescence intensity values (A) was applied to exclude probes with too low signals. A list of significant differentially expressed genes, with respect to control, was determined for each treatment with the following threshold values: a p-value for the moderated t-statistic <0.01, an FDR adjusted-p-value <0.05 and a $\log_2 A>6$.
Agilent probe sequences and IDs referring to genes not annotated at the time of microarray purchasing were extensively queried in nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ensembl (www.ensembl.org) repository to identify any newly annotated gene. Gene names were substituted to Agilent IDs only when BLAST found a 98–100% sequence identity and Ensembl confirmed the Agilent probe position in correspondence of the matching gene.

Cluster and functional annotation analysis

A two-way hierarchical cluster analysis (genes against treatments) with Euclidean distance and average linkage was performed with the three differentially expressed gene lists using the statistical JMP9.0 software (SAS Institute Inc., Cary, NC, USA).
To identify ontology as well as pathway enrichments for the differentially expressed genes, Entrez Gene IDs (www.ncbi.nlm.nih.gov/gene) of the three gene lists were loaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003)

(http://david.abcc.ncifcrf.gov/home.jsp) querying for Gene Ontology and KEGG terms (Ashburner at al., 2000). Enriched terms were considered statistically significant if the Fisher's exact probability test was<0.01 and FDR<5%.
Graphical visualization of non redundant enriched terms was obtained for each gene list by using the Enrichment Maps plugin (Merico et al, 2010) within the Cytoscape software 2.8.3 software and applying the following parameters:

Enrichment Map parameters	**Mix1**	**Mix2**	**Mix3**
p-value	0.01	0.01	0.01
FDR Q value	0.1	0.003	0.1
Overlap coefficient	0.05	0.5	0.5
Combined constant	0.5	0.5	0.5

Moreover GenMAPP (Gene Map Annotator and Pathway Profiler) application were used to designed and visualize gene expression data on maps representing biological pathways and groupings of genes.

Obesogenic effect evaluation of PCB mixtures on murine (3T3-L1) and human (SGBS) pre-adipocytes

3T3–L1 Mouse embryonic fibroblast and SGBS Human preadipocytes culture conditions and differentiation induction

Mouse Embryonic Fibroblasts (3T3-L1; ATCC® CL-173™) were gently obtained from Leo van der Ven (Institutes for Environmental Studies VU University Amsterdam, Amsterdam, Netherlands) including the culture protocol.
3T3-L1 preadipocytes were cultivated in cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), penicillin (100U/ml), streptomycin (0.1mg/ml), Non-Essential Amino Acids (Life Technologies) and subcultured with trypsin-EDTA (0.25% (w/v) Trypsin-0.53 mM EDTA) twice a week preventing cultures to reach 70% confluence to avoid overgrow that may alter their morphology and capacity to differentiate.
Cells were plated in 24-well plates at a density of 3000 cells/cm^2; after four days, when cells reach confluence, adipogenesis was hormonally induced maintaining cells for 2 days in differentiation medium DMEM high glucose

(Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1.67µM bovine insulin (Sigma) dissolved in 0.02M HCl, 1µM dexamethasone (Sigma) in ethanol 100%, 0.5mM methylisobutylxanthine (IBMX, Sigma) in 0.5M KOH and were refreshed every day. All experiments were performed in triplicates.

The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell strain was gently obtained from Prof. Dr. M. Wabitsch (University of Ulm, Germany), including the culture protocol. The cells originate from an adipose tissue specimen of a patient with SGBS.

SGBS cells were cultivated in cell culture flasks until generation 30–60 (corresponding to passage 10–20), in 0F culture medium (500ml DMEM/F12 (Life Technologies) containing 5 ml Panthotenat/Biotin (Sigma), 5 ml Penicillin/Streptomycin (Sigma)) and 10% fetal calf serum (Life Technologies).

Following, 4000 cells/cm^2 were seeded in 24-well plates for 3 days to near confluence in culture medium. Differentiation was started (day 0) by washing cells 3 times with PBS and then changing to a serum- and albumin-free differentiation medium.

According to Posovszky et al. (2008), cells were grown in Quick-Diff medium (0F culture medium supplemented with 25 nmol/l dexamethasone (Sigma-Aldrich), 0.5 mmol/l methylisobuthylxantine (Sigma-Aldrich), 0.1 µmol/l cortisol (Sigma-Aldrich), 0.01 mg/ml transferrin (Sigma-Aldrich), 0.2 nmol/l triiodotyronin (Sigma-Aldrich) and 20 nmol/l Human insulin (recombinant Yeast, Roche Diagnostics GmbH, Mannheim, Germania) called Medium 1. According to Rosenow et al., 2010 cells were grown also in a different medium with higher concentrations of several compounds that promote differentiation, called Medium 2 (0F culture medium supplemented with 38 µg/mL human transferrin (Sigma-Aldrich), 76 nM insulin Human insulin (recombinant Yeast, Roche Diagnostics GmbH, Mannheim, Germania), 380 nM cortisol (Sigma-Aldrich) and 0.76 nM triiodothyronine (Sigma-Aldrich), 1.9 mM 1-methyl-3-isobutyl-xanthine (IBMX, Sigma-Aldrich), 95 nM dexamethasone (Sigma-Aldrich).

After 4 days, media were changed, and cells were further cultured in 3FC medium: 0F culture medium supplemented with 0.1 µmol/l cortisol, 0.01 mg/ml transferrin, 0.2 nmol/l triiodotyronin, and 20 nmol/l insulin.

After 11 days, islands of differentiated cells were visible showing massive triglyceride accumulation. Experiments on 3T3-L1 were performed in triplicates, whereas Adipored assay on SGBS was performed only once (one 24 well plate (n=1) in which four wells had similar exposures).

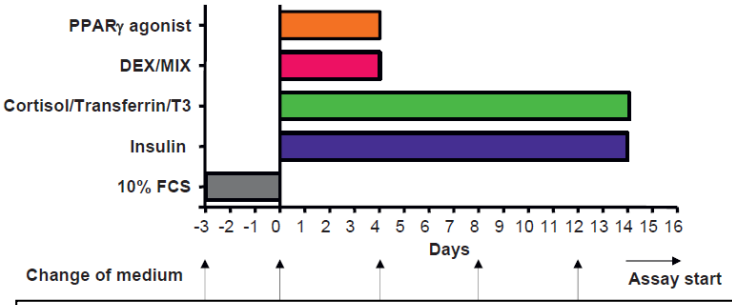


Figure 1: SGBS culture protocol (Posovszky et al., 2008

Cell lines treatments

Both 3T3-L1 and SGBS cell lines were treated with PCB once the differentiation induction started. In particular, 3T3-L1 were exposure to Mix1, Mix2, Mix3 and single PCB congeners (PCB153, PCB126, PCB118, PCB180), (concentrations indicated in Tab 1), whereas SGBGS were exposed to Mix1, Mix2, Mix3 at standard concentrations (Tab 1) as well as at concentration three times higher. Undifferentiated cells were included for both cell lines in all experiment as minimum control, moreover 0.1% DMSO (Acros, Belgium) was tested in all experiments as a vehicle control and cells treated with Troglitazone 1µM (TRO) (for both 3T3-L1 and SGBS) or Rosiglitazone (ROSI) 2 µM (only SGBS) were included in all experiments as maximum control (totally differentiated cells).
Three days after differentiation induction of 3T3 cells, medium was replaced with full culture medium containing test compounds and 1.67µM bovine insulin. Medium replacement was repeated each day until day 8 for 3T3-L1.
As for SGBS cells, four days after induction of differentiation, Quick Diff medium were replaced with 3FC Medium containing PCB mixtures that were refreshed on day 7.

Adypocyte differentiation induction evaluation

The evaluation of the effect on adipocytes differentiation exerted by PCB exposure was performed using he AdipoRed Assay Reagent (Lonza).

The differentiation of adipogenic cell lines such as the mouse fibroblast 3T3 cell line is accompanied by the accumulation of intracellular droplets of triglyceride, often used as a marker of adipocyte differentiation (Greenberger, 1979).
AdipoRed™ is a solution of the Hydrophilic Stain Nile Red that enables the quantification of intracellular lipid droplets in a high throughput manner. Nile Red, when partitioned in a hydrophobic environment, becomes fluorescent. Therefore AdipoRed™ can be used to determine the effects of test compounds on the differentiation of adipocyte precursors as characterized by the accumulation of intracellular triglycerides.
3T3-L1 cells were removed from the incubator on day 8 and allowed to cool at room temperature. The culture supernatant was removed and each well carefully rinsed with 200 µl of PBS at room temperature. AdipoRed™ Reagent (30 µl/ml PBS) was added with a multichannel pipette to each well. The plate was mixed immediately upon addition of reagent to each row of the multiwell. After 10 minutes, the fluorescence was read with an excitation wave length of 485 nm and emission at 572 nm. The fluorimeter was set to make 100 readings per well (at different points in each well), because taking the average of multiple readings per well will reduce variation between replicates.
SGBS cells were fixed on day 11 by adding 0.5 ml of Paraformaldehyde (PFA) 4% in PBS to each wells without removing supernatant, the plates were incubated 10 minutes at 4°C. Then, the supernatant was removed and other 0.5 ml of PFA 4% in PBS were added to each wells, incubating the plates 20 minutes at 4°C. Each well was carefully rinsed 2 times with 1 ml of PBS at room temperature and with 1 ml of ddH_2O. 15µl of AdipoRed™ Reagent was diluted in 15ml ddH_2O, then 1 ml of such solution was added to each well. After 10 minutes, fluorescence was read as described above.

3T3-L1 total RNA extraction and cDNA synthesis

3T3-L1 cell monolayers cultured in 6-well plates (3000 cells/cm^2) and treated as described in paragraph 3.2, were rinsed with 1ml of PBS and trypsinized; cells were then added with 350µl of RA1 lysis buffer (fournished by Total RNA isolation kit), collected and stored at -80 °C until use.
Cell lysates were extracted for their total RNA content by using the Total RNA isolation NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG**,** Düren, Germany) according to manufacturer protocol. RNA quantity was determined by NanoDrop reading (NanoDrop).

All the samples were not degradated and with an absorbance ratio at 260/280 nm≈2.
1 µg of total RNA from each sample was retrotranscribed to cDNA using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem by Life Technologies, Paisley, UK) according to manufacturer protocol.

3T3-L1 gene expression evaluation by real time RT-PCR

Real-time PCR was performed in order to assess gene expression of the following selected panel of target genes involved in adipocyte differentiation and metabolism: Hes1, Lrp5, Ncor2, Shh, Taz, Tsc22D3, Vdr, Dlk1, Cdkn1B, Ddit3. Specific primers for genes as well as for GAPDH, used as reference gene, were designed using the programme Beacon Designer 7.9 and purchased by Sigma-Aldrich. Primer sequences are listed in Tab. 4.

mm-Dlk1-3191-f	CCGAGTCTGCGAATAATTC
mm-Dlk1-3369-r	GGAGGAGTTGCTAAGAGAA
mm-Cdkn1B-1204-f	TATCGCTGACTCCATTGAA
mm-Cdkn1B-1353-r	CAACAAAGCAAATAAGGAACAA
mm-Ddit3-294-f	GGAAACGAAGAGGAAGAATC
mm-Ddit3-425-r	CTGACTGGAATCTGGAGAG
mm-Hes1-50-f	CTGGAATAGTGCTACCGAT
mm-Hes1-142-r	GTCCTTTTACTTGACTTTCATAAG
mm-Lrp5-2540-f	CGACCTCACCATTGATTATG
mm-Lrp5-2709-r	TCCAGTCAGTCCAGTAGA
mm-Ncor2-8467-f	TCACACAAGGAAGGACTC
mm-Ncor2-8662-r	CCACAGAATACGCATCAAG
mm-Shh-1970-f	ACCTTCAAGAGCCTTAACT
mm-Shh-2146-r	GCATAGCAGGAGAGGAAT
mm-Taz-1323-f	TGAACATCTGACTCCTCTG
mm-Taz-1397-r	CTTCCTTCTCCACCAAGT
mm-Tsc22D3-1121-f	TTGAGATGTGATGCCAGT
mm-Tsc22D3-1225-r	TTCTTCATAGGAACAGTCATTG
mm-Vdr-1775-f	CAGGCAGAAGAGATGAGT
mm-Vdr-1901-r	TAGGGATGATGGGTAGGT

Table 4: Primer sequences to evaluate gene expression of biomarker in 3T3-L1 by qPCR

Optimal conditions for Real-time PCR reactions were settle on in terms of amplification efficiencies and primer concentrations (Tab. 4) using the

standard curve method. Reactions were prepared using the SYBR kit and were run on a 7900HT Sequence Detection System instrument (Applied Biosystem).
Experiments were performed in duplicate on 96 well PCR plates. The thermal program was as follows: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15s, 60 °C for 45s and 95 °C for 1 min;1 dissociation cycle from 60 to 95 °C 15s to verify amplification products.
The real time-PCR amplification products were also controlled by running samples in a 2% agarose gel. Results were calculated as expDDct values corrected for the amplification efficiencies according to the Pfaffl method (Pfaffl, 2001).

Statistical analysis

Data are expressed as mean of triplicate values for each performed assay ± SEM. Data were analysed for their significance with the JMP 9.0 statistical software (SAS Institute Inc., Cary, NC, USA) by performing One-way ANOVA test and a post hoc t-test for the comparison between treated and control samples. Results were considered significant when p-value < 0.05.

RESULTS

Citotoxicity assay results

In order to an MTS assay excluded any cytotoxic effect on cell lines by PCB mixtures at the experimental concentrations used and none of the PCB mixtures tested resulted cytotoxic.

PCB mixtures exposure effect on hepatic HuH6 and HepG2 cell lines

Nuclear receptors gene expression evaluation

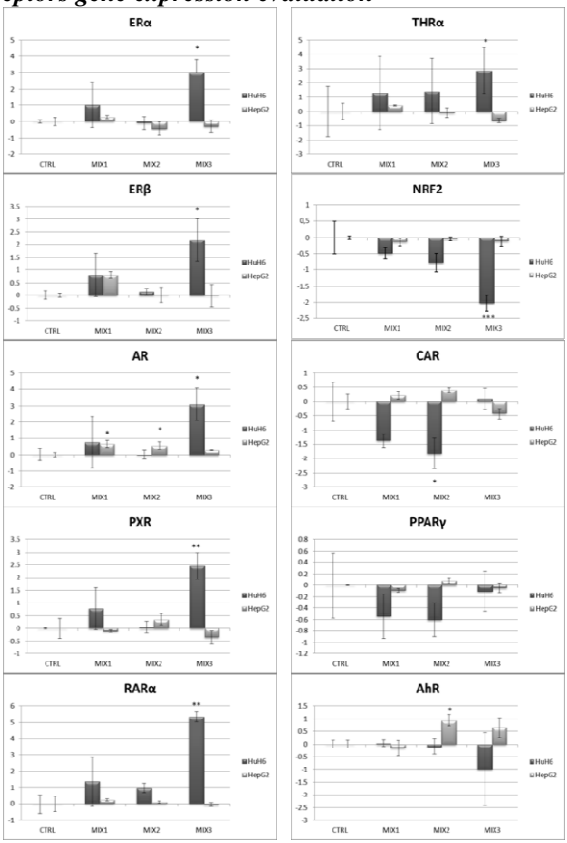


Figure 2: Gene expression modulations of NRs and NRF2 in HepG2 and HuH6 treated with the three PCBs mixtures. Values are expressed as ΔΔCt ± SEM; Statistical significance is indicated by asterisks (*=p-value <0.05; **<0.01)

HuH6 and HepG2 hepatic cell lines were differently affected by the three PCBs mixtures treatments displaying an altered gene expression modulation for almost all the NRs analysed (Fig. 2). Indeed, ERα (p-value=0.0342), ERβ (p-value=0.0339), AR (p-value=0.0527), PXR (p-value=0.009), RARα (p-value=0.0019) and THRα (p-value=0.0339) were significantly up-regulated and NRF2 (p-value=0.0009) significantly down-regulated in HuH6 by Mix3 whereas this mixture seems to not modulate the same panel of genes in HepG2.
Overall, Mix1 and Mix2, although modulating several NRs, significantly altered only a limited number of genes. Indeed, the only effect exhibited by Mix1 treatment, the other NDL-PCB mixture, was a significant increase of AR in HepG2 cells with respect to control cells. Mix2 treatment induced AR and AhR up-regulation in HepG2 as well as CAR down-regulation in HuH6. PPARγ seemed to be unaffected by both NLD- and DL-PCBs mixtures.

Cytochrome P450 activity assay in HuH6 and HepG2

HuH6 and HepG2 hepatic cell lines showed little differences in activity of Cyp3A4, CYP1A2 and Cyp2C9 metabolism enzymes, following treatments with the three PCBs mixtures (Fig. 3). Otherwise, CYP1A1 was almost null in HuH6 cells and highly expressed in HepG2 cells.

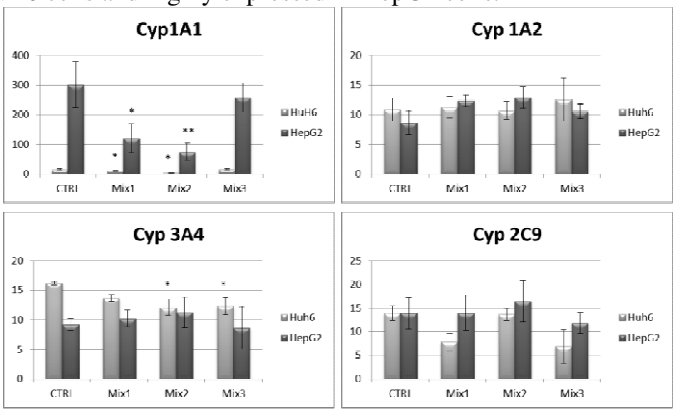


Figure 3: Activity levels of CYP enzymes analyzed in the HuH6 and HepG2 cells treated with the three PCB mixtures. Values are expressed as Luminescence Units (RLU) ± SEM. Statistical significance is indicated by asterisks (*=p-value <0.05; **<0.01)

Only Cyp1A1 and Cyp3A4 were significantly affected by the PCB mixtures. Mix1 and Mix2 significantly decreased Cyp1A1 activity levels (Mix1 p-value= 0.0405, Mix2 p-value= 0.0027) in both HuH6, despite the low detected activity, and HepG2 hepatic cell lines (Mix1 p-value= 0.0443, Mix2 p-value= 0.0177) with respect to controls. Mix 2 and Mix 3 significantly down regulated Cyp3A4 activity only in HuH6 (Mix2 p-value=0.0237, Mix3 p-value=0.03). PCB mixtures treatment didn't exert any significant modulation of Cyp1A2 and Cyp2C9 enzymes activity in both cell lines.

Oxidative stress biomarkers analysis in HuH6 and HepG2 cell lines

The evaluation of oxidative stress biomarkers in HuH6 and HepG2 highlighted differences due to PCB mixtures (Fig. 4).

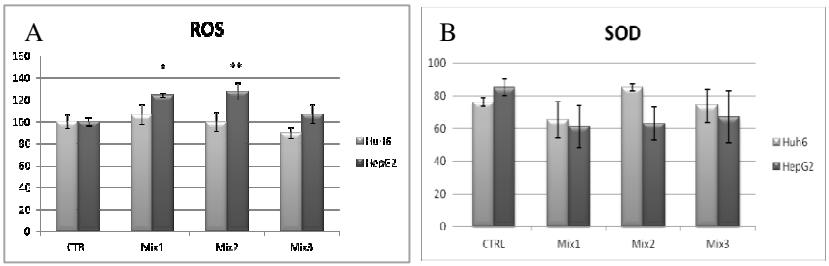
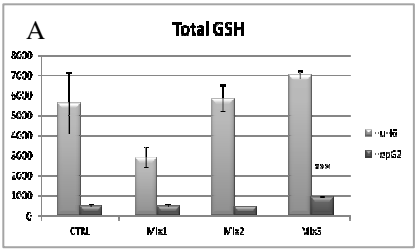
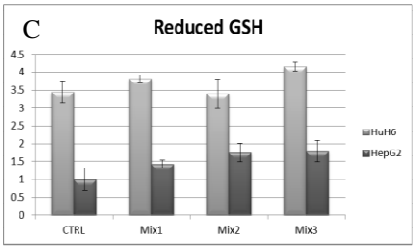


Figure 4: Analysis of ROS amount and SOD activity levels in HuH6 and HepG2 cells treated with the three PCB mixtures. Values are expressed as U/ml ± SEM for SOD activity and percentage of control (100%) for ROS levels. Statistical significance is indicated by asterisks (*=p-value <0.05; **<0.01)

Reactive oxygen species evaluation evidenced a significantly higher level in Mix1 and Mix2 treated cells, with respect to control cells, only in HepG2 cells (Mix1 p-value=0.0156, Mix2 p-value=0.0069), with HuH6 being unaffected by the three PCB mixtures. SOD activity levels were not significantly altered from PCB mixtures treatments in both hepatic cell lines.





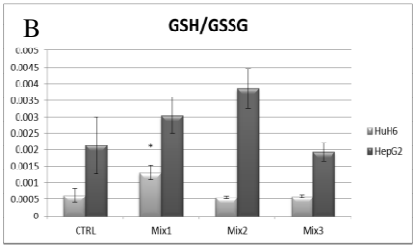


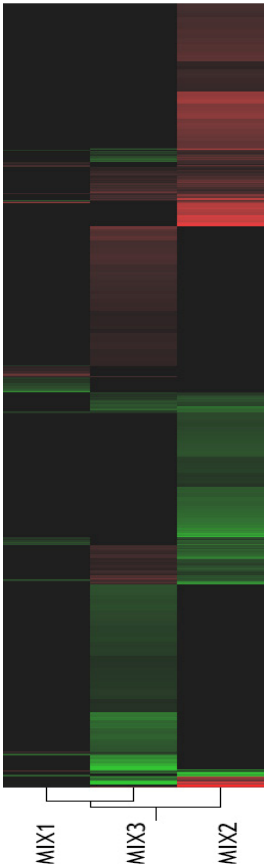
Figure 5: Analysis of total and reduced GSH amounts and GSSH/GSSG ratio in HuH6 and HepG2 cells treated with the three PCB mixtures. Values are expressed as µg± SEM. Statistical significance is indicated by asterisks (*= p-value <0.05; **<0.01)

In HepG2 a significant increase of total GSH was exerted by Mix3 (p-value=0.0009), NDL-PCB mixture. However, the GSH/GSSG ratio, considered the main indicator of oxidative stress, was increased (p-value=0.038) in HuH6 cell line by Mix1 treatment (Fig. 5)

Cell line Biomarkers	HuH6			HepG2		
	Mix1	*Mix2*	*Mix3*	*Mix1*	*Mix2*	*Mix3*
ERα			+			
ERβ			+			
AhR					+	
AR			+	+	+	
PXR			++			
CAR		-				
RARα			++			
THRα			+			
PPARγ						
Nrf2			---			
Cyp1A1	-	-		-	--	
Cyp1A2						
Cyp3A4		-	-			
Cyp2C9						
ROS				+	++	
SOD						
Total GS						+++
GSH						
GSH/GSSH	+					

Table 5: Summary of the effects exerted by the three PCB mixtures treatments for the two liver cell lines on gene expression levels of several NRs and NRF2, on the activity of four CYP450 and SOD enzymes and analytical concentration levels of ROS and GSH parameters. + represents levels significantly higher, - significantly lower respect to control cells.

Microarray profiling in HepG2 cells in response to PCB mixtures treatments



HepG2 treated with the three PCB mixtures, at the same concentrations used in biomarkers evaluation experiments, were analyzed for their gene expression profiles. Microarray data analysis revealed that the PCB mixtures modulated a large number of genes with 10450 genes altogether significantly modulated. In particular, Mix1, Mix2 and Mix3 modulated 767, 5767 and 5979 genes, respectively, with 121 genes modulated by all the three mixtures (Fig. 7) corresponding to 1.1% of total modulated genes.
Mix2 alone, featuring DL-PCB congeners, modulated 3924 genes, whereas Mix1 and Mix3, both featuring NDL-PCB congeners, uniquely modulated 326 and 4258 genes respectively. Only 99 genes were modulated by both Mix1 and Mix3, corresponding to less than a 0.9% overlap. On the other hand, Mix3 shared a higher number of genes with Mix2 (1501 genes) (Fig. 7).
The heatmap generated with the two-way hierarchical clustering (Fig. 6) revealed two quite different expression profiles in HepG2 treated with Mix2 or Mix3, although the similarity in the number of modulated genes (Fig. 7). The heatmap evidenced that in all the three mixtures, genes were up or down-regulated almost in the same number.

	MIX1	MIX2	MIX3
Up-regulated	311	3116	2873
Down-regulated	456	2651	3106
TOTAL	767	5767	5979

Figure 6: Heatmap of the two way hierarchical clustering for the 10450 genes significantly modulated by the three PCB mixtures in HepG2. Columns represent PCB treatments whereas each row represents calculated averages of modulated genes across biological and technical replica. The magnitude of modulation for each gene is indicated by shades of green or red lines standing for down-regulation and up-regulation respectively. Black lines indicate absent values.

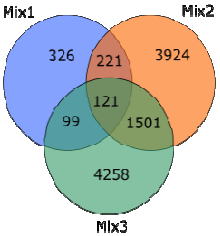


Figure 7: Venn diagrams of significantly modulated genes

Ontology terms and Pathways affected PCB mixtures treatment

A Gene Ontology (GO) and a Pathways enrichment analysis were performed through the DAVID application for each PCB-modulated gene list in order to identify possible cellular/molecular function particularly affected by the treatments.

Gene Ontology enriched terms

To better visualize non redundant enriched GeneOntology terms, the Enrichment Map plug-in within Cytoscape software (Merico et al, 2010) was used. Mix1-modulated gene list yielded top significance for terms related to metabolic processes, intracellular transport and cell organization (Fig. 8).

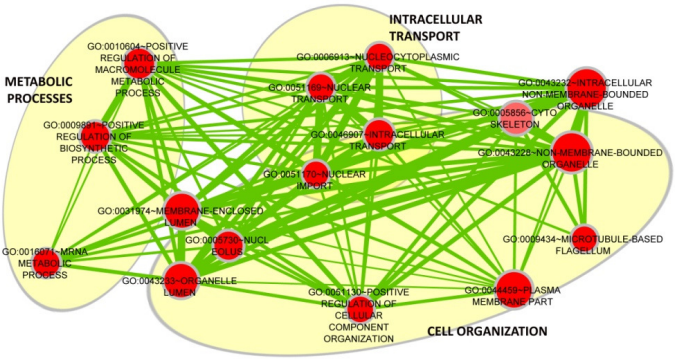


Figure 8: Enrichment Map of modulated terms (red circles) in HepG2, following treatment with Mix1. Yellow eclipses grouped enriched terms indicating cellular/metabolic processes

GO terms enriched by Mix2 treatment resulted the most significant overall, in particular those related to cellular primary processes like cell cycle and cell organization (Fig. 9). Furthermore, Mix2 enriched terms correlated with nucleotide binding processes, cell death, regulation of protein localization, protein complex assembly and protein catabolic processes, vacuoles, lysosomes and mitochondria activity, stress response/DNA repair, RNA splicing and processing. Several metabolic processes, such as those involving macromolecules, in particular proteins turnover, were also significantly altered in Mix2 treated cells.

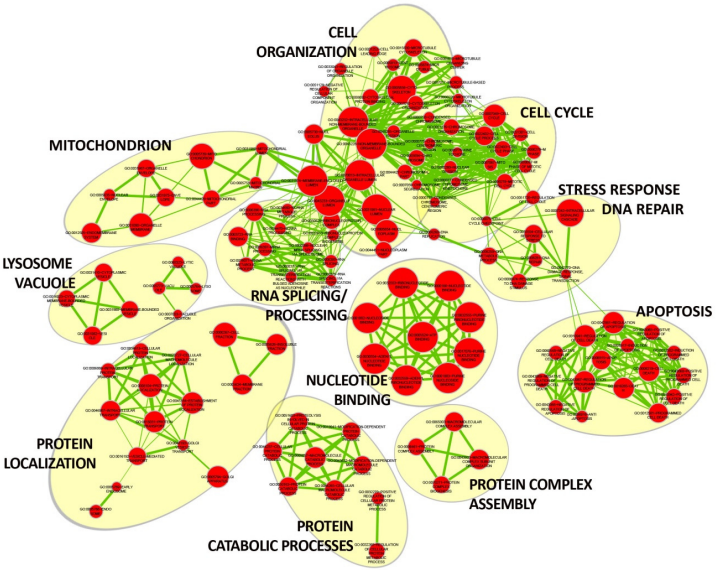


Figure 9: Enrichment Map of modulated terms (red circles) in HepG2, following treatment with Mix2. Yellow eclipses grouped enriched terms indicating cellular/metabolic processes

The top significant term for the Mix3 gene list was cell organization including regulation of cellular apparatus and organelles; Mix3 significantly altered also the expression of genes involved in regulation of transcription and RNA splicing and processing, followed by cell cycle and apoptosis, protein localization machinery, protein catabolic processes, intracellular transport, signal transduction components, cell organization and macromolecules metabolism (Fig. 10). Some terms resulted prevalently or exclusively enriched in Mix1 (cellular transport, Fig. 8), in Mix2 (mitochondria organization, stress response and DNA repair, Fig. 9) and Mix3-treated cells (signal transduction, Fig. 10), whereas others were commonly enriched by the PCB treatments, such as cell organization, regulation of transcription, protein catabolic processes and protein complex assembly.

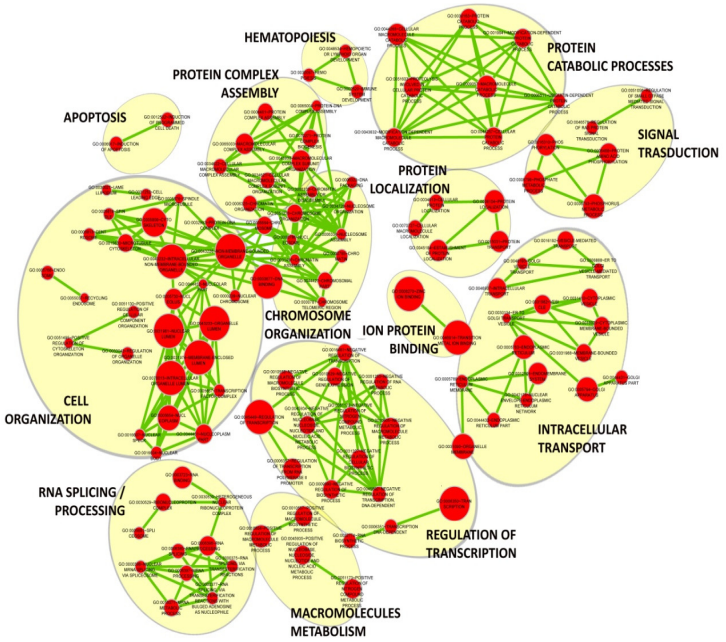


Figure 10: Enrichment Map of modulated terms (red circles) in HepG2, following treatment with Mix3. Yellow eclipses grouped enriched terms indicating cellular/metabolic processes

KEGG pathways enrichments

Pathway	FDR		
	Mix1	Mix2	Mix3
Intestinal immune network for IgA production	0.923843		
Cell cycle		9.56E-04	
p53 signaling pathway		0.030518	
Lysosome		0.034303	
Pathways in cancer		0.128852	1.830195
Spliceosome		0.18249	0.011678
Pancreatic cancer		0.355876	
Chronic myeloid leukemia		0.387217	
Prostate cancer		1.835354	0.768868
Small cell lung cancer		2.231082	
Apoptosis		2.289577	
Pathogenic Escherichia coli infection		3.29886	
Adherens junction		3.386204	0.020623
Valine, leucine and isoleucine degradation		4.235453	
Colorectal cancer		4.481132	
Ubiquitin mediated proteolysis			0.358599
Wnt signaling pathway			0.802459
Fc gamma R-mediated phagocytosis			1.501584
Endocytosis			2.113756

Table 6: Pathways significantly affected in HepG2 treated with the PCB mixtures. Values report the False Discovery Rate (< 5%) for the three gene lists as obtained by the DAVID application selecting for KEGG enrichment analysis.

Pathways analysis of PCB-modulated gene lists confirmed the results obtained with GeneOnthology enrichments. Mix1 significantly affected only the Intestinal immune network for IgA production. Mix2 affected several pathways involved in cancer and leukemia. The Mix2 impingement at cell cycle level is further supported by the significance of the apoptosis and the cell cycle pathways where the featured genes were mainly up-regulated (Fig. 11) and down-regulated respectively (Fig. 12). Mix3 uniquely affected the Wnt signaling pathway (Fig. 13) besides other pathways involved in cancer.

Mix3, although being a NDL-PCB mixture, affected similar pathways as Mix2, the DL-PCB mixtures, rather than Mix1, the other NDL-PCB mixture; indeed, both Mix2 and Mix3 affected spliceosome, some cancer pathways and adherens junctions pathways. However, as shown for the adherens junctions pathways (Fig. 14), the genes and the type of modulation exerted by the two mixtures are different.

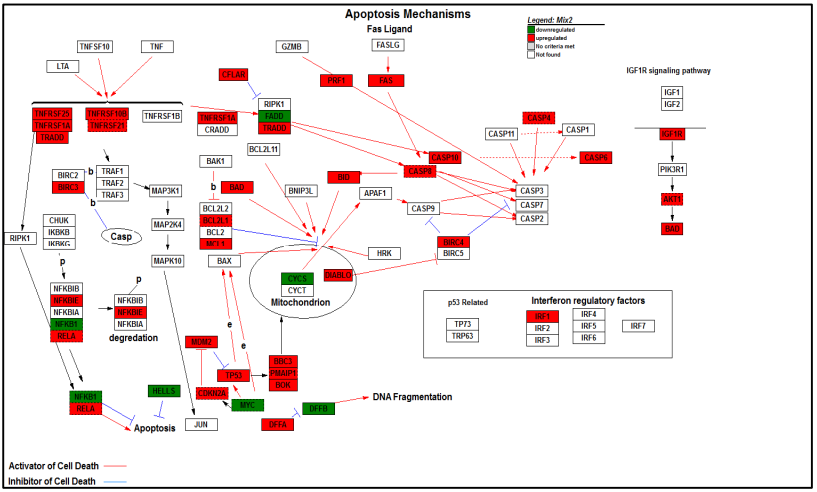


Figure 11: Apoptotic Pathway significantly affected in HepG2 treated with Mix2 obtained with GeneMAPP application. The green boxes represented down-regulated genes, the red boxes the up-regulated ones.

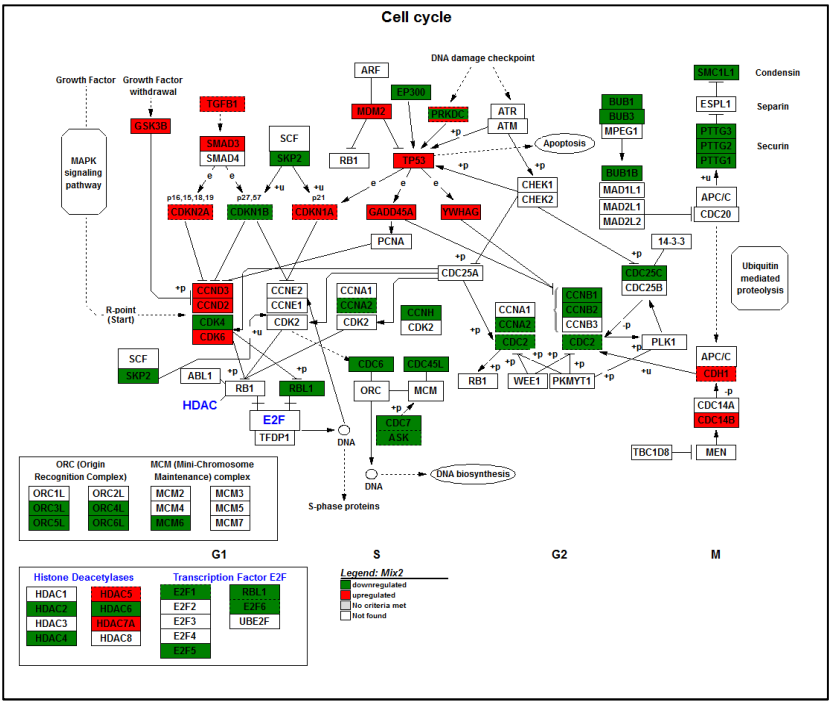


Figure 12: Cell Cycle Pathway significantly affected in HepG2 treated with Mix2 obtained with GeneMAPP application. The green boxes represented down-regulated genes, the red boxes the up-regulated ones.

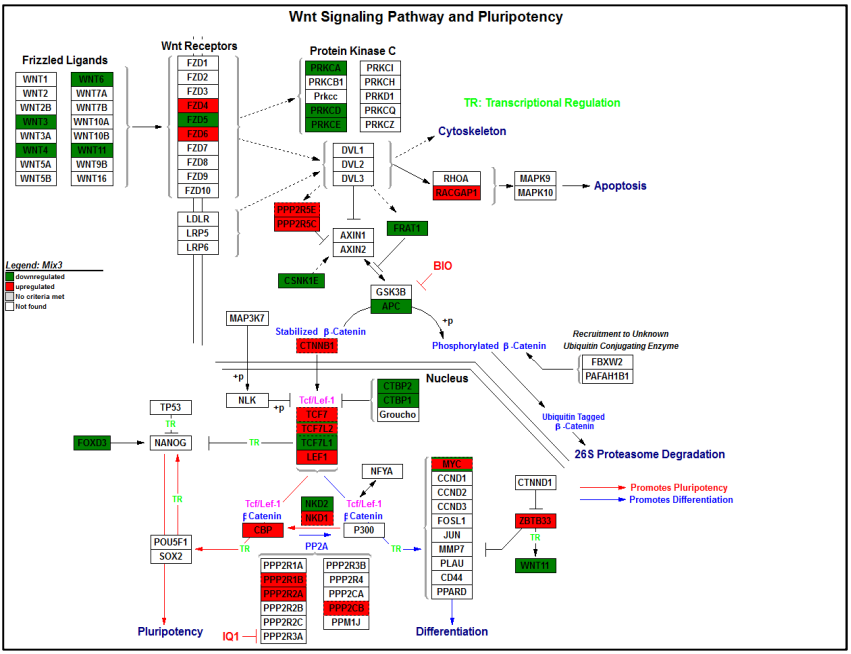


Figure 13: Wnt Signaling Pathway and Pluripotency significantly affected in HepG2 treated with Mix3 obtained with GeneMAPP application. The green boxes represented down-regulated genes, the red boxes the up-regulated ones.

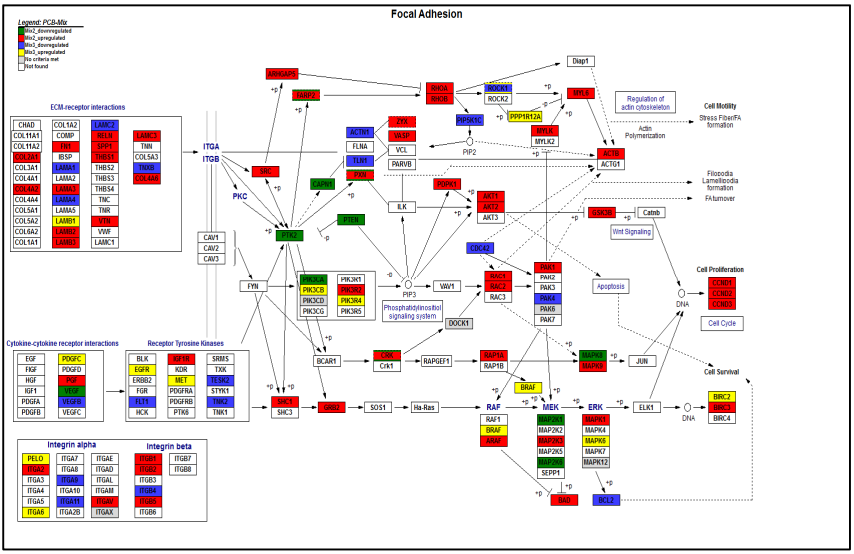


Figure 14: Focal Adhesion Pathway significantly affected in HepG2 treated with Mix2 and Mix3 obtained with GeneMAPP application. The green boxes represented genes down-regulated by Mix2, the red boxes genes up-regulated by Mix2, the blue boxes genes down-regulated by Mix3, yellow boxes are genes up-regulated by Mix3

PCB mixtures exposure effect on adipose tissue in vitro models: 3T3-L1 mouse cell line and SGBS human cell line

Evaluation of differentiation induced by PCB mixtures treatments

Adipored assay results

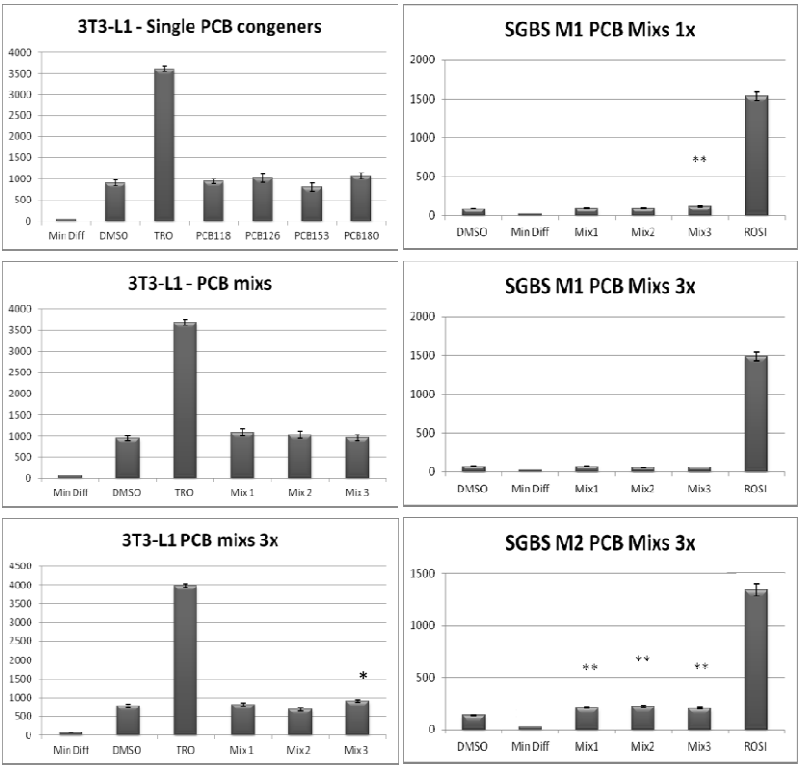


Figure 15: The differentiation of 3T3-L1 and SGBS preadipocytes treated with the three PCB mixtures. Values are expressed as relative fluorescence units RFU±SEM. Statistical significance is indicated by asterisks (*=p-value <0.05; **<0.01)

In both mouse and human (Fig. 15) cell lines representative of adipose tissue, the three PCB mixtures were tested at two different concentration: normal (1x, reflecting human internal dose) and three times higher concentration (3x). Moreover in 3T3-L1 the most abundant PCB congeners in each mix were tested at the same concentration found in the 1x mixtures. Results showed that PCB as mixtures and as single congeners did not exert any effect on 3T3-L1 preadipocytes differentiation at 1x concentration. On the contrary, 3T3-L1 differentiation resulted significantly increased after Mix3 treatment at 3x concentration (p-value=0.0266).
SGBS cells were grown in two different culturing media (M1 and M2) to determine the optimal conditions to observe differences in differentiation of cells exposed to different compounds.
In M1 at normal concentration, only Mix3 significantly affected SGBS differentiation (p-value=0.0021). SGBS cell line, grown in medium 2 (M2), showed a significant increase of differentiation after PCB mixtures treatment at 3x concentration (Mix1, p-value=0.0208; Mix2, p-value=0.007; Mix3, p-value=0.0251).
Indeed M2 resulted to induce differentiation more than M1 resulting an increased number of adipocytes after 11 days respect to preadipocytes differentiated grown in M1.
In SGBS, differentiation to adipocytes seems to be significantly affected by Mix1 and Mix2 at 3x concentration and by Mix3 at 1x and 3x concentrations.

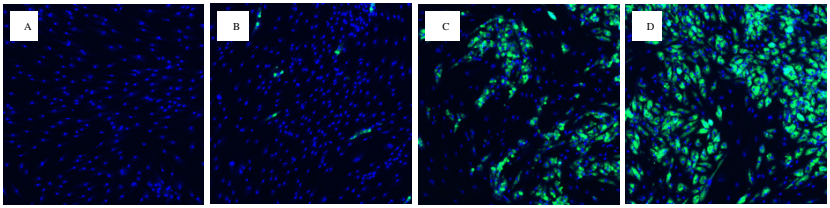


Figure 16: SGBS cells monolayer pictures captured by Arrayscan. In blue Hoechst stained nuclei, in green Nile red stained fat droplets: A) undifferentiated cells (min diff); B) cells not induced to differentiate; C) cells treated with the vehicle alone (DMSO); D) cells completely induced to differentiate through addiction of Rosiglitazone in culture medium (ROSI)

Target genes expression modulation evaluated in 3T3-L1 cells through qPCR

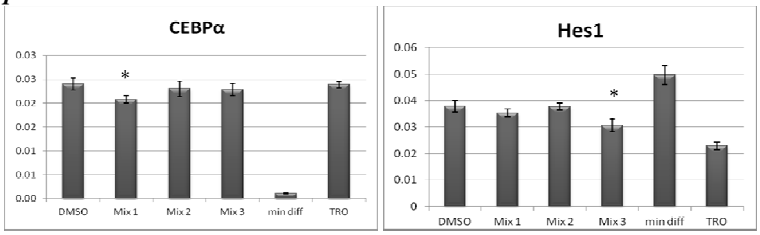


Figure 17: Gene expression modulation exerted by PCB mixtures on a panel of biomarkers. The values are expressed as expDct ± SEM. Statistical significance is indicated by asterisks (*=p-value <0.05)

Gene expression of a panel of selected biomarkers relevant in adipocytes differentiation and metabolism (Hes1, Dlk1, Lrp5, Taz, Leptin, Tsc22D3, Pparγ, Cdkn1b, Ddit3, Rxrα, Lpl, Fabp4, Vdr, C/ebpα, C/ebpβ, Igf1) were analyzed in 3T3-L1 mouse cell line following PCB mixtures treatments. One-way ANOVA and a post hoc t-test were performed to evaluate the PCB treatment effect on cells; F ratio and Prob>F were reported in table below:

Oneway ANOVA					
Gene name	**F ratio**	**Prob>F**	**Gene name**	**F ratio**	**Prob>F**
Dlk1	7.0789	0.0006*	*Ddit3*	25.6800	0.0001*
Hes1	12.1300	0.0001*	*RXRα*	0.2900	0.8700
Lrp5	10.1000	0.0001*	*LPL*	0.2200	0.9197
Taz	29.2800	0.0001*	*FABP4*	1.8700	0.1458
Tsc22d3	0.6716	0.6179	*VDR*	2.5500	0.0637
Pparγ	9.5900	0.0001*	*IGF1*	13.6300	0.0001*
Leptin	14.2229	0.0001*	*CEBPα*	0.2142	0.3308
Cdkn1b	18.7639	0.0001*	*CEBPβ*	3.6990	0.0169

T-test evidenced that the 3T3-L1 exposure to PCB mixtures did not strongly affect the selected genes. Mix1 significantly decreased C/EBPα gene expression (Fig. 17; p-value=0.0410) with respect to cells treated with vehicle alone; otherwise, Mix3 significantly down regulated Hes1 (Fig. 17; p-value=0.0145). No effect was exerted by the PCB mixtures on the other analyzed target genes (data not shown).

The TRO treatment significantly down regulated the gene expression of Hes1 (p-value= 0.0001), Dlk1 (p-value=0.0006), Vdr (p-value=0.018), Igf (p-value=0.0001) and upregulated the expression of Lrp5 (p-value=0.0002), Taz (p-value=0.0001), Pparγ (p-value=0.0001), Cdkn1b (p-value=0.0001), Ddit3 (p-value=0.0001).

DISCUSSION

Overall results confirmed that the adopted grouping of PCBs in three mixtures serves to highlight different modes of action as well as biomarkers responses, also among mixtures featuring NDL congeners. These represent new results since there are no data on PCB mixtures effects summarizing oxidative stress, metabolism, nuclear receptors gene expression and adipocytes differentiation responses following human real exposure concentrations treatment. As prompted by EFSA (EFSA, 2010), there is a need of data concerning comprehensive toxicological end-points of NDL effects. Therefore, these data may serve as a basis for developing relative toxicological factors for the NDL congeners risk assessment.

Human general population is exposed to PCBs as a sum of several congeners. Until now, extensive studies have been conducted focusing on the effects of single congener at micro-molar levels. Therefore, a new research requires the study of PCBs mixtures, because of possible additive/synergistic effects; indeed, the present study adopted "real-life" dose levels (in the pico-molar range) for each congener in the mixtures as measured in the general population (La Rocca et al., 2008) in order to mimic a realistic exposure scenario. Since PCBs have endocrine disrupting characteristics, their effects could not be translated in monotonic dose-response curves (Steinberg et al, 2011). Therefore the pico-molar range concentration, combined with the use of congeners mixture, could explain the specific effects observed in this study.

PCBs are readily absorbed from the gastrointestinal tract and accumulated in the adipose tissue. Liver is the main organ involved in metabolism, representing one of the target organs of PCBs toxicological effects (EFSA, 2010). Because liver function affects many different systems in the body, such damage could have far-reaching effects.

Due to the lipophilicity of PCBs, adipose tissue represents the storage organ of these compounds and therefore another target of PCBs action.

Therefore proper *in vitro* models originating from these two target tissues were selected to analyse PCB mixtures effects on a panel of NRs, CYP enzymes, oxidative stress biomarkers and adipocytes differentiation biomarkers. Moreover, a gene expression profiling has been performed on HepG2 cells.

Previous reports classified Mix1 as potentially estrogenic congeners (Wolff, 1995), an effect not observed in our results. On the contrary, we observed that Mix1 significantly up-regulated only AR among selected NRs in HepG2 cell line therefore suggesting a potential androgenic effect,

deserving further investigation. In HepG2 cells, we also observed an uncoupling in ROS-CYP1A1 regulation; indeed, Mix1 induced the ROS level while repressing CYP1A1 activity. CYP1A1, as well as other CYP enzymes, is an inducer of ROS production, however some evidence documented that ROS generated by one CYP can down-regulate another (Morel et al., 2000). This may occur also through CYP1A1 action itself which may decrease following ROS generation (Morel et al., 1999). We cannot explain if this unbalance of the regulatory loop induced by Mix1 occurred at CYP1A1 or ROS level also because no significant modulation of NRF2, a transcription factor which activates the expression of anti-oxidant and ROS-reducing proteins, has been observed by Real-time PCR.

In physiological conditions, both the GSSG and GSH are measurable and the concentration of GSH exceeds that of GSSG (Uys et al, 2010). In our study, GSH/GSSG ratio is significantly higher in Mix1-treated HuH6 cell line. Since no significant change was observed in GSH level, we can hypothesize that Mix1 determined a decrease of the hepatic GSSG levels. However, it has to be considered that hepatic GSH and cellular redox status are part of a dynamic process achieved by a balance between rates of GSH synthesis, GSH and GSSG efflux (Han et al., 2006).

As for Gene Ontology enrichment analysis, Mix1-modulated gene list was uniquely significant for terms related to Nuclear Transport, potentially indicating an influence on nucleus membrane trafficking; moreover Mix1 shared with both Mix2 and Mix3, the other NDL-PCB mixture, the enrichment in terms related with Metabolic processes and Cell organization.

Mix1 significantly affected only one pathway, the Intestinal Immune Network for IgA production, featuring genes of Major Histocompatibility Complex (HLA-DQA1, HLA-DQB1 and HLA-DRB4) as well as Chemokines (CCR5 e CCR10), being all up-regulated and therefore implying a Mix1 stimulation of the immune response. Overall, these molecular processes may be considered specific markers of Mix1 effect in hepatic cells since they are not exerted by the other mixtures and have not been observed in human fetal penile smooth muscle cells (hfPSMC, Tait et al, 2011).

As regards effects on adipocytes, Mix1 increased SGBS differentiation at 3x concentration, otherwise unaffecting 3T3-L1.

Among analyzed target genes involved in adipose tissue development Mix1 affected only C/EBPα being down-regulated in 3T3-L1 cells. C/EBPα is a key transcriptional regulator of adipocyte differentiation and function (Schmidt et al., 2011), which modulates leptin expression (being down-regulated by Mix1 in our model, although not significantly– data not

shown), a hormone expressed in adipose tissue with an important role in body weight homeostasis (Miller et al., 1996).
Mix2, featuring DL-PCB congeners, exerted the up-regulation of AR and AhR in HepG2 cells, confirming the well-known interaction of DL-PCBs with AhR (Safe et al., 1985). Moreover, Mix2 increased ROS levels as well as CYP1A1 mRNA expression (evidenced by Microarray analysis): both effects are mediated by AhR, as showed for TCDD (Kopf et al., 2010).
However it has to be noted that Mix2, unlike TCDD, significantly decreased the CYP1A1 enzyme activity, though the gene expression was increased, indicating a different effect possibly due to the low concentrations used.
In HuH6, Mix2 induced a down regulation of CAR mRNA expression and a reduction of CYP1A1 and CYP3A4 activity. CAR, together with PXR, recently emerged as xenobiotic sensors, is implicated in the transcriptional regulation of the CYP enzymes, therefore coupling xenobiotic exposure to oxidative metabolism (Willson and Kliewer, 2002). In particular CYP1A1 and 3A4 are indicated as CAR target genes (di Masi et al., 2009). In this respect, our results demonstrated that the decreasing of CAR mRNA expression is related to decreasing of CYP activity.
Therefore, CAR gene expression and CYP1A1 and CYP3A4 activities may be assumed as target of DL-PCBs disruption on this hepatic cell line decreasing the metabolic capability and, ultimately, the cell defense to xenobiotic exposure.
Microarray results in HepG2 cells evidenced peculiar enrichments in Mix2-modulated gene list. In particular, Mix2 affected genes involved in RNA processing/splicing, Protein localization and catabolism and genes localized in specific cellular compartments such as the mitochondrion or the lysosome. The lytic vacuole and the cytoplasmic vesicles, serve within the cells for Macromolecules degradation, which indeed resulted also a GO enriched term (Fig. 9).
Noteworthy, main and characteristic effects of Mix2 rely on enrichment of genes related to Apoptosis and to several cancer pathways (Fig. 11 and Tab. 6). One critical up-regulated gene by Mix2 is p53 which is induced by stress signals, including DNA damage, oxidative stress (Prives and Hal, 1999), apoptosis (Bates and Vousden, 1999) and tumors (Levine, 1997). The p53 protein is a transcriptional activator of genes involved in cell cycle arrest (Lanni and Jacks, 1998), cellular senescence or apoptosis (Fig. 11) but also in communication in adjacent cells (indeed Mix2 affects also the adherens junction pathway, Fig. 14). In normal cells, p53 is expressed at low level whereas it is expressed at high level in tumor cells since it contributes to transformation and malignancy. Several genes featured in the enriched cell

cycle pathway were down-regulated by Mix2 (Fig. 12) including CDKN1B (p27), an important regulator of cell cycle progression involved in G1 arrest, which inhibits G1 phase-cyclins complexes (Polyak et al, 1994). This observation is confirmed by an increased transcription of cyclins and cycline depenent kinases (CDKs) involved in G1/S phases transition.
Indeed, a further suggestion of a Mix2 inhibition of cell cycle progression, is the up-regulation of CDK6, a kinase involved in initiation and maintenance of cell cycle which interacts with D-type cyclins during interphase at G1, both (CCND2 and CCND3) resulting up-regulated as well. (Meyerson and Harlow, 1994).
Cyclins D are the first cyclins produced in the cell, in response to extracellular signals, among which the Transforming Growth Factor of β (TGFβ1) also up regulated by Mix2.
Counteracting the induction of G1 phase, Mix2 seems to inhibit S phase, since several genes playing a role in this cell cycle step were down-regulated. In particular, both RBL1 and some members of the E2F family of transcription factors involved in G1-S phase progression (De Gregori et al., 1995) were down-regulated by Mix2 (Fig. 12).
GADD45A and YWHAG, two p53-regulated proteins (Carrier et al., 1996) (Radhakrishnan et al, 2011) were up regulated. Both genes inhibit Cdc2/Cyclins B kinase activity (Hermeking et al., 1997; Vairapandi et al., 2002), which indeed were down-regulated, therefore a possible induction of G2-M arrest by Mix2 could be postulated.
p53 triggers also the apoptosis pathway, with an up regulation of several Caspase-dependent genes. It has to be noted that we did not observed neither cell death nor cell proliferation in the cytotoxicity assays; therefore, microarray results evidence early perturbation at gene expression level still not evident at cellular level.
In a previous study on same PCB mixture effects, similar modulations were observed on hfPSMC with Mix2 mainly affecting genes involved in cell cycle progression/regulation (Tait et al, 2011). Moreover, evidence demonstrated that PCB126 induced cell proliferation in Sertoli cells in adult rats (Wakui et al., 2012), whereas PCB77 causes apoptotic cell death in a dose and time dependent manner (Somiranjan et al, 2010). Since we observed either an induction of G1-phase promoting genes and of apoptosis, we may hypothesize that a sum of contrasting mode of action exerted by PCB congeners in Mix2 occurred.
As regards effects on adipocytes, Mix2 significantly induced SGBS differentiation at 3x concentration, otherwise unaffecting 3T3-L1 cells, both as mixtures and as single congeners (PCB118 and PCB126). Similarly as

for Mix1, murine and human model seemed to be differently responsive to PCB exposure. No significant modulation of target genes involved in adipose tissue development was observed in 3T3-L1 cells. Arsenescu et al., demonstrated that PCB-77, one of the congeners within Mix2, promoted 3T3-L1 adipocyte differentiation. The mixture effect or the lower concentration levels used in this study may be the cause of such different results (Arsenescu et al., 2008).
Mix3 treatment affected a wider number of NRs expression. In particular, in HuH6 we observed an up-regulation of ERα, ERβ, AR, PXR, of the two non-steroid NRs, RARα, THRα, as well as a down-regulation of NRF2.
A previous classification included PCB99 and 153, both featured in our Mix3, as having an estrogenic action (Warner et al, 2012); therefore supporting the observed ERα and ERβ induction in HuH6. To our knowledge, it is the first study that showed AR mRNA expression due to PCB congeners included in Mix3. Our results are in agreement with a previous report describing a direct activation of PXR following non-coplanar PCBs exposure in human liver cells, including PCB99, 153 and 180 featured in our Mix3 (Al-Salman et al., 2012).
A number of studies demonstrated a thyroid disrupting action by PCBs (Gilbert et al., 2012), however little is known about this mechanism which may involve also THRs. Recently a possible interaction with THRs has been postulated upon PCBs transformation to hydroxy-metabolites (Giera et al., 2011).
The observed decrease in the CYP3A4 enzyme activity in HuH6 cells was in agreement with NRF2 gene expression down-regulation (Itoh et al, 1997), however not corresponding to the PXR increase, a known CYP3 induce (Willson and Kliewer, 2002).
Total GS resulted significantly higher in treated HepG2 with respect to control cells although GSH/GSSG ratio was not affected.
Microarray analysis showed that Mix3, modulated the higher number of genes (5979 genes) with 1501 shared with Mix2-modulated genes. Apart some GO enriched terms related to Cell organization, Mix3 did not share other effects with Mix1, the other NDL mixture. Otherwise, Mix3 shared with Mix2 the enrichment of some GO terms such as those related to Protein localization, catabolism and complex assembly, as well as those implicated in RNA splicing/processing.
One of the most interesting evidence is the enrichment in modulated genes of the Wnt signaling pathway by only Mix3. β-catenin is the key activator of the Wnt-induced TCF/LEF-dependent gene expression of factors involved in cell differentiation. Mix3 up-regulated both β-catenin and three

TCF/LEF transcription factors; besides it down-regulated the APC gene which, in complex with GSK3β, phosphorylates β-catenin enhancing its degradation (Eastman and Grosschedl, 1999). Such modulations suggest an increase in citoplasmatic β-catenin stabilization that can translocate into the nucleus where it may act as coactivator of TCF/LEF family of transcription factors (Farmer, 2005). In addition, also CBP was up regulated, suggesting an unbalance toward pluripotency promotion rather than to cell differentiation (Takemarua and Moona, 2000).

Mix3 also affected some cancer pathways as well as the adherens junction pathway as Mix2 but modulating a different panel of involved genes. The adherens junction pathway in particular, was the second most significantly affected by Mix3. In a previous study only Mix2 affected hfPSMC cell-cell communication/gap junction related genes (Tait et al, 2011).

Mix2 exclusively up-regulated genes of the extra-cellular matrix (ECM), such as laminins, mainly contributing to cell attachment, differentiation and movement, as well as some integrins interacting with them. On the contrary, Mix3 exhibited contrasting effect up-regulating some and down-regulating other genes. Within the cell, integrins form adhesion complexes regulating some kinases, such as focal adhesion kinase (PTK2) and the Src family kinases, to mediate attachment to the actin cytoskeleton. While Src kinase was up-regulated by both PCB mixtures, PTK2 expression was induced by Mix3 and repressed by Mix2. PTK2 is highly expressed in cells establishing focal adhesions among cells; its down-regulation contributes to apoptosis progression and loss of focal contacts, otherwise, PTK2 over-expression leads to inhibition of apoptosis and cell proliferation observed in metastatic tumors. This further suggests a pro-apoptotic effect exerted by Mix2 and promotion in focal adhesion exerted by Mix3.

Besides PTEN, a tumor suppressor gene, was up-regulated by Mix3 and down-regulated by Mix2. This, in turn, has effect on the activation of the AKT pathway, regulating cell survival and proliferation. As a confirmation, Mix2 increased the expression of both AKT1 and AKT2 genes. The concomitant decrease of PTEN and increase of ATKs may result in a cell cycle arrest in G1 (Ramaswamy et al, 1999). However, definitive conclusion on Mix2 overall effects cannot be drawn since Mix2, other than up-regulating several pro-apoptotic genes, also induced BIRC3 and BIRC4 both members of the inhibitors of apoptosis family of proteins (IAP) (Smolewski and Robak, 2011).

Otherwise Mix3, other than promoting cell-adhesion and plutipotency through the Wnt canonical pathway, down-regulated BCL-2, while up-

regulating BRAF and BIRC2, all anti-apoptotic proteins. Therefore, also for Mix3 a final effect cannot be speculated.
Mix3 exerted the upper magnitude of effect on pre-adipocytes differentiation in both human and murine cell lines. As already observed for the other two mixtures, SGBS human model showed to be more responsive. Thus SGBS cells, treated with 1X Mix3 in M1 medium (low inducing differentiation medium), showed an increase in adipocytes differentiation. Moreover, 3X Mix3 induced SGBS differentiation only in M2 medium (fast inducing differentiation). In 3T3-L1, Mix3 exerted preadipocytes differentiation only at 3X concentration and none at 1X concentration or by the single congeners PCB153 and PCB180 suggesting a possible dose-response relationship due to a mixture effect may occur. As regards PCB153 effect on adipocytes differentiation, literature reports controversial effect. In fact our results are in agreement with previously observed lack of effect (Arsenescu et al, 2008) and in disagreement with other reported pro-differentiation effects (Taxvig et al., 2012). It has to be outlined that our study used lower concentration referred to human real exposure level. Gene expression evaluation analysis outlined that only Mix3 down regulated Hes1 a DNA binding protein whose expression blocks adipogenesis (Ross et al., 2006). Therefore, a reduction in Hes-1 expression in preadipocytes by Mix3 could promote differentiation, therefore further supporting previous evidence. In this respect, Hes1 could be considered a Mix3 biomarker of early effect. Moreover our results showed that the two mixtures of NDL-PCBs down regulated two different genes involved in adipocytes differentiation, C/EBPα by Mix1 and Hes1 by Mix3, respectively, supposing a different mode of action to be explored.

CONCLUSIONS

Overall, the selected panel of biomarkers, summarizing oxidative stress, metabolism, nuclear receptors gene expression and adipocytes differentiation, demonstrated to be a sensitive tool to highlight early effects following exposure to PCBs. The two hepatic cell lines and the two pre-adipocytes cell lines appeared to be differently reactive to PCBs, indicating the need to use different *in vitro* models and a panel of biomarkers in order to characterize the EDs effects.
Indeed the present study identified several biomarkers and processes affected by treatments characterizing the three PCB mixtures modes of action (Tab. 7) that could be recognized as early biomarkers of effect.

		Cell lines	Biomarkers
Mix1	Liver	HuH6	GSH/GSSG
		HepG2	AR, CYP1A1, ROS Nuclear Transport, Intestinal Immune Network for IgA production
	Adipose tissue	3T3-L1	C/EBPα
Mix2	Liver	HuH6	CAR, CYP3A4, CYP1A1
		HepG2	AR, AhR ROS, CYP1A1 Mitochondrion, Lysosome, Stress response DNA repair Apoptosis, Cell cycle, p53 signaling pathway, some tumor pathways
Mix3	Liver	HuH6	ERα, ERβ, AR, PXR, RARα, THRα, NRF2 CYP3A4
		HepG2	Total GS Macromolecule metabolism, Regulation of transcription Wnt signaling pathway , Ubiquitin mediated-proteolysis, Endocytosis
	Adipose tissue	3T3-L1	Hes1
		SGBS	Differentiation

Table 7: Early biomarkers of effect and processes affected by the three PCB mixtures treatments in the hepatic and adipose tissue cell lines

Moreover the selected *in vitro* models as well as the panel of biomarkers demonstrated to be a suitable and sensitive model to highlight PCB effects even at low concentrations in the pico-molar range corresponding to a real human exposure. In particular, hepatic HuH6 showed to be more responsive to PCB exposure than HepG2, regarding the effect of Mix3 on NRs modulation, and the human pre-adipocytes more susceptible than mouse cell line to PCBs treatment.

Results clearly distinguished the three mixtures indicating different modes of action especially between the two NDL-PCB mixtures (Mix1 and Mix3). In this respect, the gene expression profiling deserves a further in-depth analysis.
Finally, these data indicate the possible toxicological way to develop relative factors for the NDL congeners risk assessment.

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