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LEVELS OF POLYCHLORINATED BIPHENYLS (PCBs) AND ORGANOCHLORINE PESTICIDES (OCPs) IN BIOINDICATOR SPECIES AND EVALUATION OF TOXIC EFFECTS OF NON-DIOXIN LIKE PCBs 101, 153 AND 180 ON IMMUNE AND ENDOCRINE SYSTEMS

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Abstract

During my PhD course, the residue levels of Polychlorinated biphenyls (PCBs) and Organochlorine pesticides (OCPs) in the edible tissues of two bivalve species (*Mytilus galloprovincialis* and *Ensis siliqua*) from Tyrrhenian Sea (Mediterranean Sea) were measured. Factors explaining differences in bioaccumulation levels were also considered and an evaluation of the health risk for human consumer was provided.

A gas chromatography–electron capture detection (GC-ECD) was used for the analysis; the concentration levels of five OCPs—among which the 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane (p,p'-DDT)—and twenty PCBs, including the seven indicator-PCBs, were determined. Differences in residue levels were revealed between the two examined species; in some cases they were statistically significant. PCBs were the most abundant pollutants (mean values of 422.19 ng g^{-1} and 399.33 ng g^{-1}, respectively, for mussels and clams on fat weight), followed by DDTs, Dieldrin and hexachlorobenzene (HCB). In particular, the PCBs nos. 101, 118, 138 and 153 were the dominant congeners, accounting for 66% and 56% of the total residue levels of PCBs in mussels and clams, respectively. From the human health point of view, OCPs residue levels were below the national limits established for fish and aquatic products. Conversely, the mean concentrations of PCBs exceeded the limits set by the EU for terrestrial foods in both species.

Non-dioxin-like (NDL)-PCBs are stable and lipophilic chemicals that persist in the environment and tend to bioaccumulate in the food chain. Epidemiological studies show that PCB exposure is associated with modifications of innate and adaptive immunity, including effects on immune cells and signalling molecules involved in the response against foreign antigens. It is noteworthy that no *in vitro* studies are available regarding the impairment of immune innate response due to NDL-PCB exposure. Therefore, here we investigated the effects of NDL-PCBs 101, 153, and 180, alone or differently associated, on lipopolysaccharide (LPS)-activated J774A.1 murine macrophages. Interestingly, concentrations of the aforesaid NDL-PCBs inactive by themselves induced immunesoppression when NDL-PCBs were differently combined. In particular, the exposure to NDL-PCB mixture caused a significative suppression of LPS-induced cytokine synthesis, as well as nitrite (NO^{-2}) production and proinflammatory enzyme expression. The involvement and role of nuclear factor-kB (NF-kB) in the effects of these pollutants

was also demonstrated. Western blot analysis of NF-kB showed that LPS-induced NF-kB activation was significantly decreased by the exposure of macrophages to NDL-PCB associations. These results demonstrated the impaired capability of macrophages to respond properly to noxious stimuli, such as LPS, mimicking the environmental co-exposure to these compounds. In conclusion, our findings suggest that, although less toxic than dioxin like (DL) congeners, the NDL-PCBs tested are equally dangerous as well as immunotoxic pollutants, also considering their presence as mixtures and at higher levels than DL-PCBs in biotic and abiotic matrices.

Interestingly, NDL PCBs tend to accumulate in adipose tissue. Therefore, we evaluated the alteration of the mature 3T3-L1 adipocyte metabolism induced by PCB 101, 153 and 180 alone or associated two by two or all together. We observed an increase in lipid content and leptin gene expression and a concomitant reduction of hormone receptor expression and activity. These modifications support the induction of leptin-resistance, a typical metabolic complication of obesity. Consequently, we investigated how these PCBs affect the expression of important proteins involved in the signalling of leptin receptor. In particular, the phosphatase PTP1B and the suppressor of cytokine signalling (SOCS) 3, two negative regulators of leptin signalling, were induced by the association of the PCB 153 with the 180 or of all PCBs. Conversely, the same associations caused a significant decrease in the phosphorylation of STAT3, a downstream activator of the transcription of leptin gene targets. This effect has been also associated to the inactivation of AMPK/ACC pathway through the reduction of the phosphorylation of these enzymes, and hence the increase in lipid content. Furthermore, it was highlighted the ability of these pollutants to increase the transcription of inflammatory cytokines, such as IL-6 and TNFα. Interestingly, it is important to highlight that PCB concentrations used in this study are comparable to levels detectable in human adipose tissue. Our data strongly support the hypothesis that these substances may interfere with the lipid metabolism contributing to the development of obesity and related diseases.

Obesity is a clear risk factor for Osteoarthitis (OA). Adipokines are factors, dysregulated in obesity and that play an increasing pathogenic role in OA. Apoptosis is involved in extracellular matrix (ECM) degradation, thus the identification of inductors of this process is important for the understanding pathogenesis/progression of OA. We also evaluated the adverse effect of PCBs 101, 153 and 180 on human and murine chondrocytes by assessing

apoptosis pathways. Murine chondrogenic ATDC5 cell line and human T/C-28a2 immortalized chondrocytes were exposed to NDL-PCBs 101, 153 and 180. Cell viability was examined using MTT assay. Necrosis was evaluated by LDH assay and Annexin V flow cytometric assay. Expression of apoptotic related proteins, such as caspase 3, Bcl-2 and Bax, was assessed by Western blot analysis. Oxidative stress was evaluated by malondialdehyde (MDA) assay and the Oxidative Stress Index (OSI). Exposure to examined PCBs caused the loss of cell viability and accelerated apoptosis in a concentration-dependent manner both in murine and human chondrocytes. Data from Annexin V and LDH assays showed necrosis induction. Caspase 3 activation, as well as, altered Bcl-2/Bax ratio and p38 phosphorylation suggested apoptosis induction. Finally, MDA levels and OSI revealed that PCBs drive chondrocytes towards oxidative stress. Our results indicate that the activity of PCBs on cell viability is likely to be mediated by alterations in the mechanisms of regulation of apoptosis and necrosis. Overall, these data highlight a novel role of environmental pollutants in the pathophysiology of chondrocytes.

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Chapter 1

1. INTRODUCTION

During the last decades many organochlorine compounds (OCs), such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), have been employed as inputs in the Agriculture and Industry sectors, determining increased amounts of these environmental pollutants in biotic and abiotic matrices of ecosystems. Despite attempts to control the spread of these dangerous pollutants, implemented through the adoption of preventive measures [for polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)], bans on production and use (for PCBs), problems caused by this situation are still complex to resolve. Interestingly, for areas not particularly affected by industrial emissions, the presence of these compounds in the environment is caused not by current emission but by persistent detection of these contaminants in so-called "environmental reserves".

In fact due to high chemical stability and lipophilicity, OCs tend to persist in the environment for a very long periods and to bioconcentrate as well as to biomagnify in the food chain (Porte and Albaiges, 1993). Improper procedures for the disposal of municipal waste and the occurrence of conditions, which predispose the persistence and the bioaccumulation of the aforesaid environmental pollutants, such as waste incomplete combustion, represent also a well known source of persistent organic pollutants (POPs), thus causing a plethora of noxious effects on human health.

It is noteworthy that POPs are responsible of chronic toxicity, involving alterations of several physiological functions and inducing adverse effects which are not often accompanied by evident symptoms. Accordingly it’s not always possible to perform an adequate recognition of their hazard and an assessment of human exposure levels.

PCBs, in addition to well-known PCDDs and PCDFs, belong to the group of POPs: they are chlorinated organic compounds widely distributed in the environment. PCBs have been used on a large scale for the first time in the '20s and their production in the industrial sector reached its peak in the '60s, resulting in a state of environmental emergency. In studies on humans exposed to PCBs, noxious effects on neurological functions, endocrine (like dioxins, they are known as "endocrine disruptors") and reproductive or immune systems have been observed (El-Shahawi et al., 2010).

To date despite the adoption of restrictive rules, the exposure continues mainly through: diet and, in particular, through the consumption of contaminated fish and other animal or vegetable origin foods, where these compounds tend to bioaccumulate; inhalation of volatile PCBs derived from industrial products prior to the ban and, finally, groundwater or soil contamination, since they have been found at high levels, due to their long half-life, in these abiotic matrices.

The major source of PCB exposure for humans is represented by the ingestion of contaminated foods, particularly those of animal origin. Occupational exposure affects only a limited slice of the population, but accidental exposure rarely occurs. Indeed the daily human exposure is, in 90% of cases, due to contaminated foods (90% of animal origin, especially fish and dairy products) (Fattore et al., 2008).

Mixtures of PCBs are generally evaluated on the basis of chemical analysis where the properties of seven PCBs, that have been defined indicators (iPCBs; IUPAC nos. 28, 52, 101, 138, 153 and 180), are taken as reference by the European Union (EU). These compounds are considered to be markers of pollution caused by all PCBs. iPCBs are the predominant congeners in biotic and abiotic matrices (Weijs et al., 2006) and biomagnificate in human diet. The sum of 6 iPCBs (excluded the PCB 118 which is a

dioxin-like congener, DL) accounts for the 50% of total non dioxin like (NDL) congeners in food (European Food Safety Authority, 2005). Although NDL-PCBs are less toxic than DL congeners, these pollutants are equally harmful since they have been most commonly detected, in blood and tissues of humans, wildlife and fish exposed via the food chain to environmental PCBs (Ferrante et al., 2010) and at higher concentration than the latter congeners (Storelli and Perrone, 2010).

Because of their lipophilicity, PCBs and PCDDs, tend to concentrate mainly in adipose tissue. Moreover, it is now more clear that their chemical stability contributes to their environmental persistence as well as to their bioaccumulation and biomagnification in different animal species (Burreau et al., 2006). The latter phaenomenon determines the persistence of these pollutants in various environmental compartments with the achievement of the highest concentrations in tissues of several animal species at the top of the food pyramid and humans.

During my PhD, the assessment of bioaccumulation of PCBs and OCPs in two mussel species from Campania Region was carried out. Although various PCBs and OCPs have been banned in the EU during the 1970s and 1980s, contamination of coastal areas of Mediterranean countries is still observed. Actually, large amounts of OCPs and PCBs reside in coastal waters, estuaries, and coastal sediments (Carro et al., 2010), suggesting that the aquatic environment can be considered a reservoir for these organochlorines.

About bivalve molluscs, evidence are available for mussels, usually suggesting the presence of consistent levels of pollutants in fresh and marine waters (Carro et al., 2010; Licata et al., 2004; Porte and Albaiges, 1993; Villeneuve et al., 1999). Being a filter feeder and sedentary organism, with wide geographical distribution (Carro et al., 2010), the mussel is an useful biomonitor which provids temporal and spatial variations of OCs in

aquatic systems, and informations to implement an hazard analysis for human consumer. Conversely, studies on clams are lacking.

In Italy, evidence about OC contamination levels in sea species, mainly regard the Adriatic Sea (Bayarri et al., 2001; Di Muccio et al., 2002; Perugini et al., 2004), show the presence of high residue levels. About the Tyrrhenian Sea, high contamination levels are reported for the aquatic ecosystems of the Campania region (Ferrante et al., 2007; Ferrante et al., 2010; Naso et al., 2005).

In the present study we analysed Mediterranean mussel (*Mytilus galloprovincialis*) and razor clam (*Ensis siliqua*) from the coastal area of Castelvolturno, in the Campania region. As noted by fishery sector operators, razor clams have recently experienced a steep decline in Adriatic and Tyrrhenian areas, which might be due to natural and anthropogenic factors, such as exploitation and pollution by OCs.

The amounts of some OCs, such as the 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane (p,p'-DDT), hexachlorobenzene (HCB) and PCBs, were measured in edible tissues of mussels and clams, and differences in the accumulation pattern of the various pollutants among the two species were evaluated. The goal was to identify a correlation between the OCs residue levels and factors influencing the bioaccumulation, such as the distribution of the organochlorines in the abiotic environmental components of bivalves habitat. Moreover, to have a gauge of the relative significance of contamination level in the study area, our results were compared to those obtained in other Italian regions or countries. Finally, an evaluation of the health risk for consumer and the species analysed was provided. In this study, we not only evaluated the OCs bioaccumulation, but we also investigated the effects of NDL-PCBs 101, 153 and 180, alone or differently associated, on

immune and endocrine systems. In fact these pollutants are currently considered as endocrine disruptors by the scientific world (El-Shahawi et al., 2010).

The physico-chemical properties and toxic effects of PCBs are strictly structure-dependent. Indeed, ortho-substituted NDL-PCBs, with a low affinity for the Aryl hydrocarbon Receptor (AhR), have been known to affect several signal transduction systems and cause neurotoxicity as well as immune suppression in fish, birds and mammals.

On the basis of several immune toxicological investigations, these pollutants have been argued to induce immune suppression, reducing antibody production (Selgrade, 2007) and phagocytosis (Levin et al., 2005a). *In vivo* studies in rodents or primates exposed to PCBs or dioxins have obtained results similar to those of clinical studies, confirming the suppression of antibody responses to subsequent immunizations (Park et al., 2008).

Regarding their immunotoxicity in humans, PCBs are considered as probable causal factors involved in the pathogenesis of non-Hodgkin lymphoma, other lymphatic/haematological cancers and breast adenocarcinoma (Falck et al., 1992).

Several epidemiological studies have shown that PCBs cause alterations in both innate and adaptive immunity, interfering with the immune response and its induction. Consequently, an increased incidence of infections, a reduced antibody response and alterations in lymphocyte function and distribution have been found. Indeed exposure to PCBs, assessed in term of concentration levels in blood withdrawals from the umbilical cord, in maternal serum and breast milk, has been associated with an increased incidence regarding respiratory and ear infections, influenza and chickenpox in healthy Germans (Weisglas-Kuperus et al., 2004), in Inuit preschool children (Dallaire et al., 2006), in children of workers employed in capacitors, especially in those breastfed for long periods,

and in children exposed before birth through the ingestion of rice oil contaminated with PCBs and PCDFs during the environmental disasters of Yushi and Yu-Cheng (Chao et al., 1997; Guo et al., 2004; Yu et al., 1998). Several clinical and epidemiological studies have investigated the correlations between PCBs exposure and the reduction of immune cell function and the delayed-type hypersensitivity (Bilrha et al., 2003).

Reduction in the number of cells that mediate the early immune response, including monocytes, polymorphonuclear cells and natural killer (NK) cells, may affect the ability of the immune system to recognize xenobiotic antigens and limit the transduction of immune signals induced by acute cytokine and chemokine. This process is necessary for the beginning of a complete and functional immune response. Although studies on NDL-PCBs are lacking, recently our research group has shown that NDL-PCBs suppress macrophage vitality and, in particular, PCBs 101, 153 and 180 induce macrophage apoptosis (Ferrante et al., 2011), confirming previous data that have shown that exposure of human healthy leukocytes to PCBs 138, 153 and 180 decreases phagocytosis by suppriming activity of both neutrophils and monocytes (Levin et al., 2005a). Exposure to PCB 169 and to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), however, does not affect phagocytosis, suggesting a different pattern for NDL-PCB mediated immunotoxicity (Levin et al., 2005b). Although epidemiological studies on the effects of PCBs on immune system are few, toxicological studies evidence PCB effects on the inflammatory process (Strauss and Heiger-Bernays, 2012). Data on the mechanism of action show that DL-PCBs act in the acute phase of inflammatory response, when the macrophages recognize patterns of foreign biological substances through the activation of Toll-like Receptors (TLRs) that trigger different signal transduction pathways, leading to the production of proinflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF) α and

macrophage inflammatory protein (MIP)-2 (Kerkvliet, 2009). Although the mechanism of AhR-mediated immunotoxicity for DL-PCB congeners has been determined, our recent research has also shown that also NDL-PCBs have immunotoxic effects (Ferrante et al., 2011). Several indirect mechanisms, such as the influence on immune-neural axis, mediated by interference with calcium homeostasis, or binding to serotonin receptors have been proposed (Duffy-Whritenour et al., 2010).

Since the endocrine and immune systems share portions of some intracellular signaling pathways, NDL-PCBs, recently recognized as endocrine-disrupting chemicals (EDCs), may be considered potential toxic agents for influencing host defense system against foreign pathogens.

In the present study, we investigated the effects of NDL-PCBs 101, 153 and 180, alone or in combination (100 nM), on lipopolysaccharide (LPS)-induced cytokines synthesis, such as tumor necrosis factor (TNF) α, interleukin (IL)-6 and monocyte chemoattract protein (MCP)-1, NO^{-2} production, inducible nitric synthase (iNOS) and cyclooxygenase (COX)-2 expression and nuclear factor (NF)-kB activation in the J774A.1 macrophage murine cell line.

Interestingly, concentrations of PCBs inactive by themselves caused immunosuppression when PCBs were combined. Indeed our results revealed that exposure to NDL-PCB mixtures caused a significative suppression of LPS-induced cytokine synthesis, as well as NO^{-2} production and proinflammatory enzyme expression. Western blot analysis of NF-kB showed that LPS-induced NF-kB activation was significantly diminished by the exposure of cells to the associations of the aforesaid pollutants.

These results clearly demonstrate that NDL-PCB mixtures impaire capability of exposed macrophages to respond properly to noxious stimuli, such as LPS, by interfering

with NF-kB activation. Thus, NDL-PCBs might play important roles in the inflammatory response and host defense system against foreign pathogens, since they suppress the immune innate response.

PCBs are immunotoxic compounds but they also tend to accumulate in lipid-rich tissues of organisms that contaminate, because of their high lipophilicity. There is increasing evidence in the literature correlating the exposure to polychlorinated biphenyls (PCBs) with the diffusion of obesity and obesity related disorders, such as type II diabetes (Everett et al., 2011; Lee et al., 2011; Silverstone et al., 2012). Elevated serum concentrations of PCBs have been related with the onset of metabolic syndrome, glucose intolerance and cardiovascular diseases (Airaksinen et al., 2011; Ha et al., 2007; Uemura et al., 2009). This correlation was supported by experimental *in vivo* and *in vitro* studies evidencing that NDL-PCBs impair glucose homeostasis in lean and obese mice (Baker et al., 2013), induce adipocyte differentiation and promote obesity in rats (Arsenescu et al., 2008). This evidence suggest a key role of these chemicals in the fundamental mechanisms involved in the control of adipose tissue metabolism. In particular, the "environmental obesogen hypothesis" postulates that the PCBs, as well as other endocrine disrupting chemicals, may interfere with the lipolysis pathway, the triglyceride synthesis and the adipocyte differentiation, as well as may increase the expression levels of diverse enzymes implicated in lipid metabolism or transcription factors regulating energy homeostasis in fat cells (Arsenescu et al., 2008).

PCBs tend to accumulate in lipid-rich tissues of the organisms they contaminate, due to their highly lipophilic character. The adipose tissue (AT), therefore, constitutes one of the most significant internal reservoir of these POPs (Müllerová and Kopecký, 2007) and, together with other human specimens, such as breast milk and serum, it has been used

in biomonitoring the extent of human exposure to OCs (Arrebola et al., 2012; Shen et al., 2012; Yu et al., 2011). The AT plays a significant role in kinetic and toxicity of PCBs. This role is complex and might seem paradoxical. Indeed, there is evidence that AT is protective under conditions of acute or subacute exposure to PCBs as well as to other POPs. Storage in the lipid droplets has a buffering effect and prevents the persistence of high serum levels of these POPs and their detrimental effect on other more sensitive lipophilic tissues, such as brain.

Recent epidemiological studies support a role for PCBs in modulating the mechanisms of energy balance control (Dirinck et al., 2011). Modulating AT differentiation, metabolism and function, these pollutants could affect not only the physiological role of AT, but also influence the development of obesity-associated diseases (Casals-Casas and Desvergne, 2011; Schug et al., 2011).

Overall DL-PCBs are a relatively minor component of the total body burden in human serum (Cave et al., 2010). Conversely, NDL-PCBs, that are prevalent in the environment, but remain largely overlooked in relation to their toxic effects on AT functions (Crinnion, 2011).

Epidemiological and mechanistic studies are necessary to investigate all aspects of NDL PCB toxicity in order to confirm or modify the Maximum Residue Limits, recently established by EU legislation (EU Commission Regulation 1259/2011), referred to the sum of six indicators (iPCBs) (PCB 28, 52, 101, 138, 153, 180) proposed by EU as markers of PCB contamination. These limits are of great toxicological interest, being fixed to protect consumer health from exposure to foods containing PCBs. Experimental studies have been mainly performed to evaluate the accumulation dynamics, storage and release in/from

adipose cells of NDL PCBs (Bourez et al., 2013; Bourez et al., 2012; Gallenberg et al., 1987).

The majority of functional studies were focused on DL PCBs or PCB 153, one of the most abundant NDL PCBs found in humans and environment. However, to date there are no in vitro studies on the effects of other NDL PCBs (such as PCB 101 and PCB 180), or their combination on adipocyte function or metabolism. Several reasons explain our interest about these congeners. First, PCBs 101, 153, and 180 are three of six iPCB congeners; second, they are the most frequently detected; third, they are revealed at high concentrations in human tissues, including adipose tissue (Corsolini et al., 1995; Duarte-Davidson et al., 1994; Malarvannan et al., 2013) and food of animal origin (Domingo and Bocio, 2007; Törnkvist et al., 2011; Ferrante et al., 2010).

Here, we have investigated the effect of these NDL PCBs alone or mixed on mature 3T3-L1 adipocytes by assessing lipid content and leptin sensitivity. Among adipokines, leptin, synthesized and released proportionally to fat mass content (Kallen and Lazar, 1996), plays a pivotal role in maintaining adipose tissue balance/stabilization. In particular, we have investigated PCBs effect on leptin (ob) and leptin receptor expression (ObRb), evaluating cell responsiveness to the hormone through the analysis of Janus kinase (JAK) 2-Signal Transducers and Activated Transcription (STAT) 3 signalling cascade. Moreover, the effects of PCBs on lipid metabolism and storage have been also determined through the modulation of the pathway AMP kinase/Acetyl CoA carboxylase downstream leptin receptor signalling.

NDL-PCBs are, therefore, POPs that accumulate preferentially in adipose tissue causing a low grad inflammation and endocrine disruption. Obesity is a clear risk factor for Osteoarthitis (OA). In fact OA has a pathogenesis involving adipokines, particularly leptin

(Scotece et al., 2013). However, only few studies have analyzed the correlation between exposure to these pollutants and the pathogenesis of OA. It is known only that subjects recruited after the incident of "Yusho" in Japan, which had been accidentally exposed to high levels of PCBs and PCDFs through the ingestion of rice oil contaminated, showed joint swelling and arthralgia. In the cohort of subjects recruited to "Yucheng" in Taiwan, after accidental exposure to PCBs, there was, however, an incidence of arthritis four times higher than that seen in subjects not exposed (Chao et al., 1997; Guo et al., 2004; Yu et al., 1998). Recently, it has been suggested a positive correlation between the levels of PCB exposure and the incidence of arthritis in women. The alteration of the mechanisms of programmed cell death (apoptosis) is known to be related to the degradation of the extracellular matrix (ECM) in the cartilage of patients with OA. Chondrocytes are the only cells present in the articular cartilage and preserve the integrity of the cartilage itself. Thus, the cell death of chondrocytes is responsible for damage to the cartilage by the appearance of which depends on the onset of OA itself. Apoptosis of chondrocytes is observed, among other things, more frequently in advanced cases of OA that in normal subjects. Since apoptosis is a critical event in the pathogenesis of OA, the identification of inducers of this process is considered a key element in understanding the pathogenesis and/or progression of OA.

Since PCBs have been associated with the incidence of OA and with the induction of apoptosis in different cell lines, here it has been hypothesized that PCBs 101, 153 and 180 induce the apoptotic process in chondrocytes resulting in cartilage damage and was determined the involvement in the production of oxidative stress in the promotion of apoptosis.

2. PHYSICOCHEMICAL PROPERTIES OF PCBs AND OCPs

2.1 Polychlorinated biphenyls (PCBs)

PCBs (Fig. 1) are internationally recognized as compounds belonging to the group of POPs, together with PCDDs and PCDFs. PCBs are a family of man-made organic chemicals with a common structure that vary primarily in their degree of chlorination [Agency for Toxic Substances and Disease Registry, (ATSDR), 2000]. These compounds are characterized by two benzene rings linked by a C-C bond, with up to 209 congeners characterized by the general formula $C_{12}H_{(10-n)}Cl_n$ (1=n=10) and a molecular weight between 189 and 499 $g \cdot mol^{-1}$ (Behnisch et al., 1997).

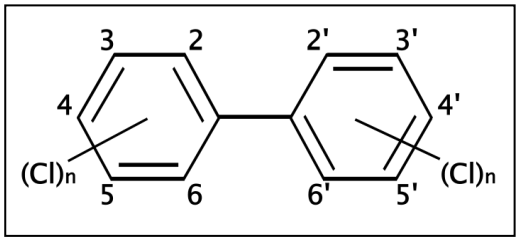


Fig. 1 General molecular structure of polychlorinated biphenyls with the numbering system. In PCBs, some or all of the 10 hydrogens (attached to carbon atoms numbered 2–6 and 2'–6') are substituted with chlorines.

For their classification it has been generally used the International Union of Pure and Applied Chemistry (IUPAC) nomenclature or, since the system proposed by the IUPAC was too complicated to be applied, it has been proposed another system proposed by Ballschmiter and Zell (1980). This last system provides that PCB congeners were numerated from 1 to 209. This nomenclature system is used until today. For example, PCB 126 is named according to IUPAC rules as 3, 3', 4, 4', 5-pentachlorobiphenyl.

It should be noted that while PCBs and furan compounds are deliberately produced chemicals, dioxins are unwanted by products of several chemical processes, such

as combustion. PCBs in pure form are odourless or mildly aromatic solids or oily liquids, often found in mixtures with other organic chemicals (ATDSR, 2000). Generally all congeners show a low solubility in water (negative associated to the chlorination degree; Table 1), a fair solubility in organic solvents and high lipophilicity, which promotes the high environmental persistence and bioaccumulation (Magnusson et al., 2006).

Table 1. Some physicochemical properties of PCBs at 25 ºC

Homologous groups	Molecular weight (g mol^{-1})	Chlorination degree (%)	$Pv^{298.15}$ (Pa)	Pk_{ow} (-Log K_{ow})	S (µg L^{-1})
Mono-CB	188.7	18.8	2200-920	4.5-4.7	1300-7000
Bi-CB	223.1	31.8	370-75	5.0-5.6	56-790
Tri-CB	257.6	41.3	110-13	5.6-6.1	15-640
Tetra-CB	292.0	48.6	18-4.4	5.9-6.7	19-170
Penta-CB	326.4	54.3	5.3-0.88	6.4-7.5	4.5-12
Hexa-CB	360.9	58.9	1.9-0.2	7.1-8.3	0.44-0.91
Hepta-CB	395.3	62.8	0.53-0.048	7.9	0.47
Octa-CB	429.8	66.0	0.078-0.009	8.4-8.6	0.18-0.27
Nona-CB	464.2	68.7	0.032-0.011	9.1	0.11
Deca-CB	498.7	71.2	0.0056	9.6	0.016

$Pv^{298.15}$= Calculated vapour pression at 25° C; S= water solubility at 25° C; Pk_{OW}= partition coefficient water/octanol

Because of their physicochemical properties, the enzymatic degradation is extremely difficult. In addition, the strong lipophilic character of PCB increases the risk of bioaccumulation in humans and biota.

The difference in the chemical and physical properties as well as in the biological effects between the congeners is directly related to the degree of chlorination, the substitution pattern of the molecule and its structure. The phenyl rings, in fact, can rotate around the bond that connects them but this rotational freedom is limited by the position of the chlorine atoms.

If only the meta and para positions are substituted, the two rings lie in the same plane, and in this case, we obtain the co-planar PCBs that have a toxicity dioxin-like and generally higher than the non-coplanar congeners. Indeed, twelve non-ortho and mono-ortho PCBs, that may take on co-planar conformations like PCDD/PCDF, are called dioxin-like (DL)-PCBs.

Conversely, if at least one ortho position is substituted, because of the steric hindrance brought by the atom of chlorine, the two rings lie on different planes and perpendicular to each other. Thus we have the non-coplanar PCBs, which are considered, generally, less toxic than DL-PCBs even if, on the other hand, are present in higher concentrations in the environment. These congeners are usually called non dioxin-like (NDL)-PCBs.

Both the DL- and NDL-congeners are very stable to changes in pH, oxidation and photo degradation. PCBs are also non-flammable (if the number of chlorine atoms is greater than 4) and little volatile; these compounds have an evaporation temperature above 800° C and decompose only above 1000° C.

Characteristics such as low dielectric constant, the density greater than water, the high lipophilicity make them unreadily biodegradable. For their physicochemical properties, PCBs were widely used in a variety of industrial equipment (e.g., electrical, heat transfer, and hydraulic equipment) and consumer products (e.g., plasticizers in paints, plastics, and rubber products), although manufacturing of PCBs was stopped in the U.S.A. in 1977 and worldwide by the Stockholm Convention (Table 2; Xu et al., 2013).

Table 2. POPs listed in the Stockholm Convention 2001 amendment.

Item	Pollutants	Type	Isomers and homologues
2001 amendment	"dirty dozen" list		
1	Aldrin	Pesticide	Aldrin and isodrin
2	Dieldrin	Pesticide	_
3	Endrin	Pesticide	_
4	Chlordane	Pesticide	α- and β-isomers
5	Heptachlor	Pesticide	_
6	HCB	Pesticide and industrial	_
7	Mirex	Pesticide	_
8	Toxaphene	Pesticide	Hundreds of isomers
9	DDT	Pesticide	p,p'-DDT; o,p'-DDT; p,p'-DDE; p,p'-DDD
10	PCBs	Industrial and by-product	209 congeners
11 and 12	PCDDs and PCDFs	By-product	75 PCDD congeners and 135 PCDF congeners

2.1.1 NDL-PCBs 101, 153 and 180

Mixtures of PCBs are generally assessed on the basis of a chemical analysis of seven PCBs (PCB-28, 52, 101, 118, 138, 153 and 180). These compounds are often referred to as "indicator" (i)PCBs and their total concentrations are indicative of contamination with PCBs (Storelli and Perrone, 2010). iPCBs occur in highest concentrations in both biotic and abiotic matrices and are routinely determined in environmental laboratories around the world (Babut et al., 2009). Indeed, the sum of six indicator PCBs (congeners 28, 52, 101, 138, 153 and 180) represents about 50% of the total NDL-PCBs in food (EFSA, 2005).

In the present work we analyzed PCB 101, 153 and 180 (Fig. 2), which are three of the seven iPCBs identified by ICES as markers of the degree of environmental contamination due to PCBs. From the PCB 101/153 up to 180, the number of chlorine

atoms increases progressively and, in the same time, there is an increase of lipophilicity as well as an increment of their capability to persistence in the environment and to storage in the adipose tissue.

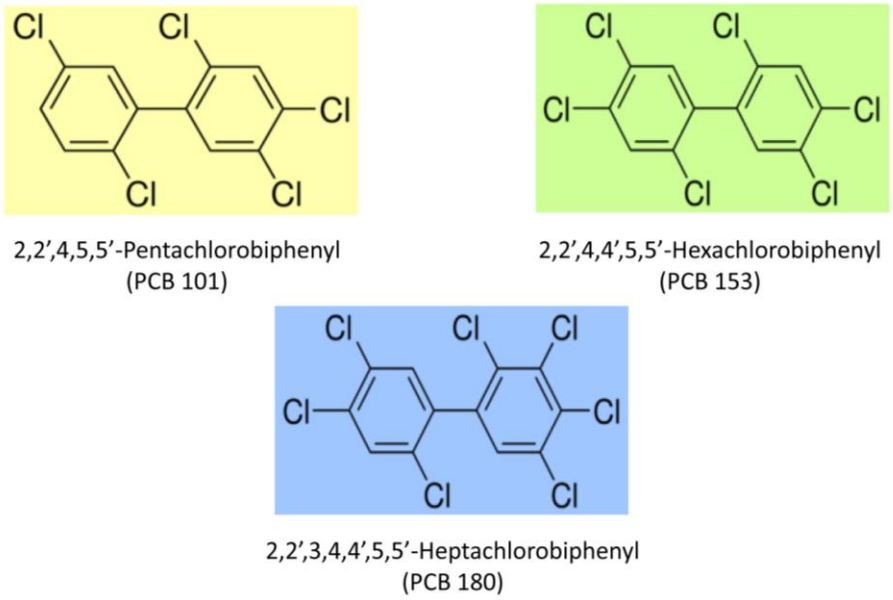


Fig. 2 PCB 101, 153 and 180 structures and their IUPAC nomenclature.

2.2 Organochlorine pesticides (OCPs)

Organochlorine pesticides (OCPs) are amongst the most commonly used pesticides in developing countries because of their low cost and broad spectrum of activity against various pests. OCPs on the initial Stockholm Convention (SC) list in 2001, also called "dirty dozen" list, include aldrin, chlordane, 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), dieldrin, endrin, heptachlor, mirex, toxaphene and hexachlorobenzene. Interestingly, although the use of these compounds have been banned globally under the SC on POPs, these pollutants are still detected in biotic matrices because of their bioaccumulation in nature. These pesticides not only tend to accumulate in adipose tissue but also biomagnify through food chain due to their lipophilic nature and long half-lives

(Wang et al., 2012). They have a wide range of acute and chronic health effects like cancer, reproductive disorders, immune suppression, congenital defects, and endocrine dysfunction (Alvarez-Pedrerol et al., 2008; Dewan et al., 2013).

2.2.1 Hexachlorobenzene

Hexachlobenzene (HCB, Fig. 3) is a chlorocarbon with the molecular formula C_6Cl_6. HCB is a lipophilic and persistent pesticide that has been used to treat seeds of sorghum, wheat and other grains against fungi.

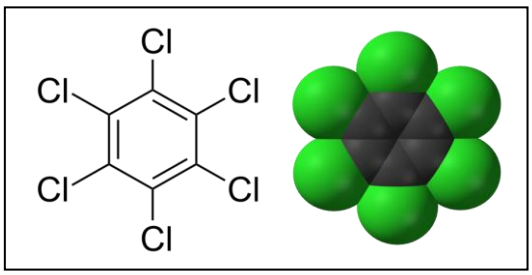


Fig. 3 HCB structure and three-dimensional conformation.

HCB is a white crystalline solid that has negligible solubility in water and variable solubility in different organic solvents. It is most soluble in halogenated solvents like chloroform less soluble in esters and hydrocarbons, and even less soluble in short chain alcohols. Its vapour pressure is 1.09×10^{-5} mmHg (1.45 mPa) at 20°C. Its flash point is 242°C and it sublimes at 322°C.

HCB has been classified by the International Agency for Research on Cancer (IARC) as a Group 2B carcinogen (possibly carcinogenic in humans). Although concerns about the adverse effects on the environment and human health resulted in the discontinuation of the use of HCB as a pesticide in many countries during the 1970s, human chronic exposure to low HCB doses is still a concern due to the persistent nature of this OCP. Animal carcinogenicity data for HCB show increased incidences of liver cancer

(Abdo et al., 2013). Moreover, HCB has also been found to impair neurobehavioral functions in rats after pre- and post-natal exposure (Lilienthal et al., 1996).

In humans, HCB has been shown to cause damage to the liver, thyroid, nervous system, bones, kidneys, blood, and immune and endocrine systems (Reed et al., 2007). Furthermore, a previous research suggests that exposure to low levels of HCB and other OCPs can affect young children's neurological and behavioral development (Stewart et al., 2000). HCB may also cause teratogenic effects. Human and animal studies have demonstrated that HCB crosses the placenta to accumulate in foetal tissues and is transferred in breast milk. HCB is very toxic to aquatic organisms (Shen et al., 2008; Nakashima et al., 1999). Ecological investigations have found that the risk of bioaccumulation in an aquatic species is high and biomagnification within the food chain frequently occurs (Goutte et al., 2013).

2.2.2 Dieldrin

Dieldrin (Fig. 4), a chlorinated hydrocarbon, is an insecticide and a by-product of the pesticide Aldrin. From 1950 to 1974, dieldrin was widely used to control insects on cotton, corn and citrus crops. Also, dieldrin was used to control locusts and mosquitoes, as a wood preserve, and for termite control. Usually seen as a white or tan powder, most uses of dieldrin were banned in 1987, however, dieldrin is no longer produced in the United States due to its harmful effects on humans, fish, and wildlife. Dieldrin is a persistent, bioacculumative, and toxic pollutant targeted by the United States Environmental Protection Agency (U.S. EPA). Dieldrin is closely related to aldrin, which reacts further to form dieldrin. Aldrin is not toxic to insects; it is oxidized in the insect to form dieldrin which is the active compound.

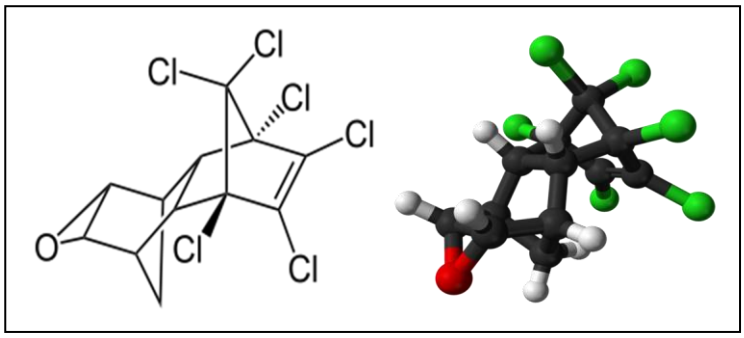


Fig. 4 Dieldrin structure and three-dimensional conformation.

Both dieldrin and aldrin are named after the Diels-Alder reaction which is used to form aldrin from a mixture of norbornadiene and hexachlorocyclopentadiene. Furthermore this insecticide tends to biomagnify as well as to biamagnify within the food chain (Zhao et al., 2013). It has been linked to diseases such as breast cancer (Boada et al., 2012) and to noxious effects on immune, reproductive and nervous system (Schaalan et al., 2012).

2.2.3 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DTT)

DDT is a colorless, crystalline, tasteless and almost odourless OC known for its insecticidal properties. Being highly hydrophobic, it is nearly insoluble in water but has good solubility in most organic solvents, fats and oils.

This pesticide is produced by the reaction of chloral (CCl_3CHO) with chlorobenzene (C_6H_5Cl) in the presence of sulfuric acid as a catalyst. In 1949, DDT was first introduced for a malaria vector control trials and was simultaneously used for agricultural pest control. In 1983, its use in agriculture was banned, except in emergency. Commercial DDT is a mixture of several closely–related compounds. The major component (77%) is the 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethane (*p,p'*-DDD), the *p*-isomer, which is shown in Fig. 5.

DDT is converted in the environment to other more stable forms, including *p,p'*-DDD and 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p'*-DDE). Similar to parent

compound its degradation products are highly persistent in the environment, they bioaccumulate and may undergo biomagnification within food chains (Loflen, 2013).

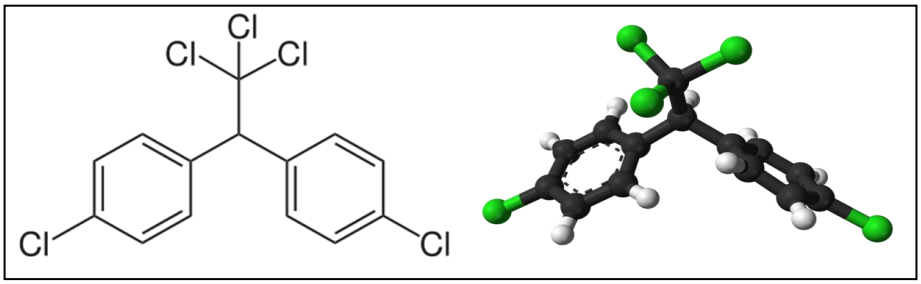


Fig. 5 p,p'-DDD structure and three-dimensional conformation.

3. MECHANISM OF ACTION

3.1 PCBs

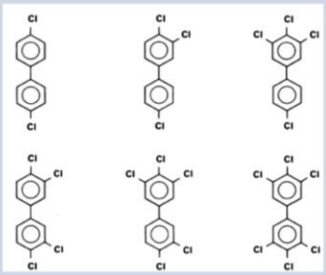
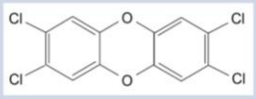
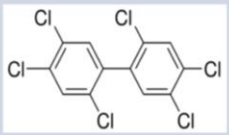
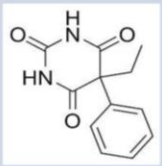
The toxic effects of PCBs depend on the positions of chlorine atoms and consequently on the steric structure of the molecule. The most noticeable difference in mechanism of action is found due to the presence or absence of chlorine atoms on the *ortho* (2, 2', 6, 6') positions. Those PCBs that do not present *ortho* chlorines and have two pairs of adjacent chlorines on the *meta* and *para* positions can have high-affinity binding to the Aryl hydrocarbon receptor (AhR; e.g., PCBs 77, 126, and 169). Both the biochemical and the toxicological modes of action have a conspicuous resemblance to those of the PCDDs and PCDFs (Safe, 1992).

With an increasing number of *ortho* chlorines, the possible planar configuration of the PCB molecule becomes increasingly more difficult because of the steric hindrance. As a result, the binding affinity of these (multiple) *ortho* PCBs to the AhR decreases drastically. In this group of *ortho*-substituted congeners, only some mono-*ortho*-substituted PCBs (e.g., PCBs 105 and 118) exhibit some binding to the AhR, which results in dioxin-like toxicity and biochemical effects (Safe, 1994). Conversely, PCB congeners that possess two or more *ortho* chlorines (e.g., PCB 153) do not exhibit any significant dioxin-like toxicity due to the lack of relevant binding to the AhR.

Notably the most distinct difference between various groups of PCB congeners is found in the way they induce cytochrome P450 (CYP) enzymes. Those congeners that show high-affinity binding to the AhR can be potent inducers of the CYP1 family, including CYP1A1, CYP1A2, and CYP1B1, which resembles the induction by 3-methylcholanthrene. In contrast, those PCBs that have an *ortho* substitution pattern induce

enzymes from the CYP2 and CYP3 families, which resembles the induction by phenobarbital. Due to this activity, this group of PCBs is often referred to as "Phenobarbital-like PCBs". In this respect, the mono-*ortho*-PCBs take an intermediate position, as they can induce enzymes from both the CYP1 family as well as the CYP2 and CYP3 families (Safe et al., 1992; 1994).

Table. 3 PCBs: Summary of structure-funcion relationships.

PCB structures	Cytochrome Induction resembles
Coplanar PCB congeners substituted at both *para* and 0-4 *meta* positions 	2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)  3-methylcholanthrene (3-MC) 
Mono- and Di-*ortho*-analogues of coplanar PCBs	Mixed type of induction pattern
Non coplanar PCBs 	Phenobarbital 

Only coplanar PCBs, similar in structure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), elicit their toxic effects through agonism of the AhR; whilst non coplanar PCBs are not ligands for AhR, but may be ligands for members of the nuclear receptor family of proteins, such as Pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Ludewig et al., 2007).

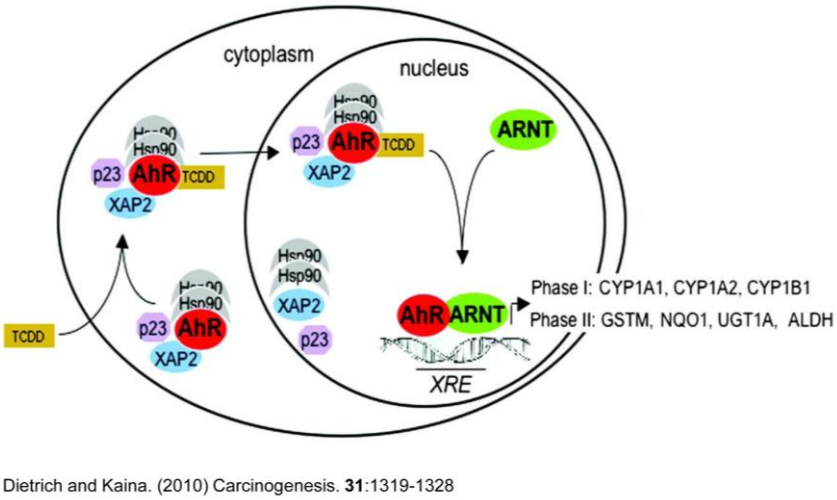
The AhR is a ligand-dependent basic helixloop-helix-Per-ARNT-Sim (PAS)-containing transcription factor that forms a nuclear heterodimer with the AhR nuclear

translocator protein to activate gene transcription (Denison et al., 2011). The AhR responds to exogenous and endogenous chemicals with the induction/repression of expression of a plethora of genes and production of several biological and toxic effects in a wide range of species and tissues (Beischlag et al., 2008; Furness and Whelan, 2009; Hankinson, 2005; Humblet et al., 2008; Marinkovic´ et al., 2010; White and Birnbaum, 2009).

The best-characterized high-affinity ligands for the AhR include a wide variety of ubiquitous hydrophobic environmental contaminants such as the halogenated aromatic hydrocarbons (HAHs) and non halogenated polycyclic aromatic hydrocarbons (PAHs). Among the HAHs, the TCDD was the first compounds identified as an AhR ligand (Bigelow and Nebert, 1982; Poland et al., 1976). TCDD exposure and bioaccumulation can produce diverse species- and tissue-specific biological and toxic effects, including tumor promotion, teratogenicity, immuno-, hepato-, cardio-, and dermal toxicity, wasting, lethality, modulation of cell growth, proliferation and differentiation, alterations in endocrine homeostasis, reduction in steroid hormone–dependent responses, and induction and repression of expression of a large number of genes.

AhR complex-mediated gene expression involves interaction with dioxin responsive elements (DREs) in 5'-promoter regions of target genes, and the overall mechanisms of this response have been intensively investigated using the CYP1A1 gene and other drug-metabolizing enzymes as models (Whitlock, 1999). More recent studies demonstrate that this genomic pathway for activation of the AhR is more complex than the classical induction of CYP1A1, and there is also evidence for extranuclear activity of the AhR (Huang and Elferink, 2012; Tanos et al., 2012). As shown in Fig. 6, the AhR, at cytosolic level, exists in an inactive state as a multiprotein complex containing heat shock

protein 90 (hsp90), HBV X–associated protein 2 (XAP2), and the co-chaperone protein p23 (Beischlag et al., 2008; Marlowe and Puga, 2010).



Dietrich and Kaina. (2010) Carcinogenesis. **31**:1319-1328

Fig. 6 The classical mechanism of AhR-dependent gene activation. The cytosolic AhR is complexed by two molecules of Hsp90, XAP2 and the co-chaperone p23. Binding of a ligand, e.g. TCDD, leads to a conformational change, thereby allowing nuclear translocation of the AhR complex. In the nucleus, the AhR dissociates from the complex and dimerizes with ARNT. The AhR–ARNT heterodimer then binds to xenobiotic-responsive elements (XREs) in the promoters of genes encoding for several phase I and phase II metabolizing enzymes but also several other genes, e.g. CYP2S1, COX2 or Slug. GSTM, glutathione-S-transferase M; NQO1, NAD(P)H:quinone oxidoreductase 1; UGT1A, uridine 5′-diphosphate-glucuronosyltransferase 1A; ALDH, aldehyde dehydrogenase.

Following ligand binding, the AhR is presumed to take in a conformation change that exposes its N-terminal nuclear localization sequence, facilitating translocation of the ligand-AhR complex into the nucleus. Once in the nucleus, binding of the AhR to a structurally related nuclear protein called ARNT (Ah receptor nuclear translocator) results in progressive displacement of hsp90 and other subunits from the AhR complex, facilitating additional AhR:ARNT interactions and conversion/transformation of the ligand:AhR:ARNT complex into its high affinity DNA-binding form (Hankinson, 1995; Soshilov and Denison, 2008). Binding of the heterodimeric ligand AhR:ARNT complex to its specific DNA recognition site, referred to as a dioxin-responsive element (DRE), xenobiotic-responsive element, or Ah-responsive element, upstream of a responsive gene (such as CYP1A1 and other AhR-dependent–responsive genes), leads to DNA bending,

coactivator recruitment, chromatin and nucleosome disruption, increased promoter accessibility to transcription factors, and increased rates of gene transcription (Beischlag et al., 2008; Hankinson, 2005; Whitlock, 1999).

The presence of an AhR complex in a wide variety of species and tissues and its ability to act as a ligand-dependent transcription factor has suggested that many of the toxic and biological effects of AhR ligands result from differential alteration of gene expression in susceptible cells. Additionally, the ability of metabolically stable AhR agonists (i.e., TCDD and DLCs) but not metabolically labile AhR agonists (i.e., PAHs and related compounds) to produce the spectrum of AhR-dependent toxic effects also has suggested that the overt toxicity of selective AhR ligands results from their ability to persistently activate/repress expression of key AhR responsive genes. Consistent with this hypothesis are the results of studies using transgenic mice in which the AhR, or selected functions of the AhR, or ARNT have been disrupted (Bunger et al., 2003, 2008; Gonzalez and Fernandez-Salguero, 1998). These and other studies demonstrated an absolute requirement of the AhR and ARNT in the ability of TCDD to produce its major toxic and biological effects (Bunger et al., 2003, 2008).

The qualitative and quantitative composition of PCB mixture, the different affinity for the AhR, the concentration of the above mentioned pollutants, the type of exposure and the biological variability drive the activation of genes' transcription.

Furthermore, PCB congeners can be divided into two major groups, namely DL- and NDL-PCBs. DL-PCBs have one or no chlorine atoms at the *ortho*-positions and adopt a coplanar structure and, as a consequence, their toxic effects are similar to those reported for dioxins. Most of the *ortho*-substituted PCBs evokes, on the other hand, different responses not linked to interaction with the AhR, including the decrease in the levels of

dopamine, the alteration of the levels of thyroid hormones and the binding to the estrogen receptor (Safe, 1990).

Some PCBs showed estrogenic or androgenic activity in vitro (Andersson et al., 1999; Arcaro et al., 1999). Many of these compounds therefore can mimic sex steroid activities and are, therefore, potential endocrine disruptors giving increased risk of reproductive disorders and carcinogenesis. *In vivo* and *in vitro* studies have shown that selected PCBs, their metabolites and/or mixtures are capable of mimicking some of the biological activities of estrogens including precocious puberty, disrupted uterus and induction of estrogen responsive enzyme activities (Bitman and Cecil, 1970; Jansen et al., 1993; Safe, 1994; Li and Hansen, 1996). Although different results have been obtained about the effects of PCBs on the development of the gonads, depending on the animals and congeners considered, generally, PCBs and their metabolites are known to interfere with sexual determination and differentiation (Qin et al., 2005).

Mechanistic *in vitro* effects have been reported, such as competition with 17β-estradiol (E_2) for binding to ERα and ERβ, promotion of proliferation of the human breast cancer cell line MCF-7, and induction of gene expression (Vonier et al., 1996; Arcaro et al., 1999). Based on their chemical structure and activities, it has been suggested that those PCB congeners that possess *ortho* substituents can elicit estrogenic responses following hydroxylation at a vacant *para* position (Korach et al., 1988; Gierthy et al., 1997). However, in addition to hydroxylated metabolites (Connor et al., 1997), non-*ortho* substituted PCB congeners (Nesaretnam et al., 1996) and selected parent PCB congeners may also exhibit estrogenic and antiestrogenic activities (Nesaretnam et al., 1996; Fielden et al., 1997; Nesaretnam and Darbre, 1997; Lind et al., 1999). Moreover PCB mixtures show a synergistic effect on estrogenic activity (Arnold et al., 1996).

3.1.2 NDL-PCBs

NDL-PCBs have at least two chlorine substitutions at the *ortho*-positions on the biphenyl ring that twists the structure away from a single plane. This significantly reduces the affinity for AhR, and indeed these compounds are likely to act as inhibitors of AhR-mediated activation (Suh et al., 2003).

For this reason NDL-PCBs have shown a toxicity profile very different from that of TCDD. Particularly, *in vivo* studies showed that PCB 153 is able to activate the nuclear factor (NF)-kB, even after a single administration (Lu et al., 2003), and that the deletion of the p50 subunit of this factor inhibits its carcinogenic activity.

It is becoming increasingly clear that NDL-PCBs are more likely to act as ligands for members of the nuclear receptor family of transcription factors (Wu et al., 2009). It has been suggested that these congeners may act as ligands for CAR and/or PXR (Jacobs et al., 2005; Schuetz et al., 1998), and may thus activate CAR/PXR target genes expression.

This activity may be also important considering that not all of the adverse effects associated with PCB exposure are mediated via AhR. For example, the hydroxylated metabolites of PCBs are weak estrogen receptor agonists, as well as inhibitors of estrogen sulfotransferase; as such, PCBs may have an endocrine disrupting effect, which may underlie their reproductive toxicity (Kester et al., 2000).

Furthermore, NDL-PCBs have been recently shown to bind to the pregnane X receptor (PXR) in the nucleus of mammalian cells (Al-Salman and Plant, 2012). Upon interaction with a ligand from a variety of endogenous or exogenous chemicals, PXR forms a heterodimer with the retinoid X receptor (RXR) and binds to the xenobiotic response elements (XREs) located in the promoter regions of the target genes, thereby regulating their transcription. As shown in Fig. 7, these target genes encode three phases of

xenobiotic metabolizing enzymes, typically, CYP3A4, UDP glucuronosyl transferase (UGT) 1A1 and multidrug resistance (MDR)-1 p-glycoprotein.

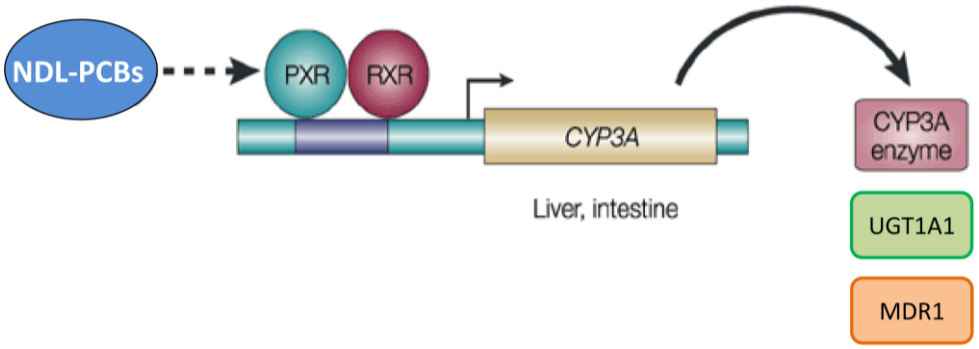


Fig. 7 The molecular basis of NDL-PCB binding to pregnane X receptor (PXR). PXR is a transcription factor that regulates the expression of the CYP3A, UGT1A1 and MDR1 genes in the liver and intestine. It functions as a heterodimer with the nuclear receptor RXR. NDL-PCBs bind to PXR and induce expression of downstream target genes. CYP, cytochrome P450; UGT1A1, glucuronosyl trnsferase 1A1; MDR1, multidrug resistance p-glycoprotein 1; PXR, pregnane X receptor; RXR, retinoid X receptor.

3.2 OCPs

OCPs share many characteristics with PCBs, such as the high lipophilicity and the elevated capability to persist in the environment due to their accumulation in biotic matrices and food chain especially in the species at the top level.

Most of scientific data correlates human chronic exposure to these pollutants with the occurrence/incidence of cancer, reproductive defects, neurobehavioral abnormalities, endocrine and immunological toxicity. As reviewed by Mrema et al. (2013), they show these effects through several mechanisms, including disruption of endocrine system, oxidation stress and epigenetic modulation. The high chlorination degree of OCPs is responsible of their chemical inertness and fairly rigid conformation. As a consequence, these compounds can’t usually undergo enzymatic degradation but more frequently exert their toxic effects due to agonistic or antagonistic binding to the intracellular receptors for which natural hydrophobic substances, similar to themselves in structure, are the

endogenous ligands, such as steroid hormones. Agonistic binding leads to recruitment of coactivators and thus increases transcriptional activity while antagonistic binding prevents coactivator recruitment and/or attracts corepressors, leading to decreased transcriptional activity of the receptors.

OCPs therefore are known or suspected to be endocrine disrupting chemicals (EDCs) likewise other POPs. Endocrine activity of OCPs can be due to direct binding with hormone receptors, because of their conformational similarity with the receptor-binding portions of natural hormones, mainly of the steroid groups. This is the case of aromatic polychlorinated substances such as DDT.

As shown in Fig. 8, they most commonly can also interfere with the binding of steroid hormones, such as estradiol (E_2), to their nuclear receptors (NRs) due to the interaction with the AhR. This receptor is a key regulator of the cellular response to xenobiotic exposure (Swedenborg et al., 2009) and its activation is very similar to NRs (Wu and Koenig, 2000). It is noteworthy that EDCs can interfere with hormone system due to mechanisms that are based on the cross-talk between NR and AhR signalling (Swedenborg et al., 2009).

Firstly, as ligands of AhR, they can target hormone receptors to the proteasome, such as earlier findings have shown about ERα degradation (Wang et al., 1993). Furthermore, since AhR and NR are known to share transcriptional co-activators, EDCs can disturb steroid hormone signalling, competing with endogenous NR ligands for the recruitment of common co-activators.

Finally, two other mechanism, which mediate endocrine disruption, have been described: the first one consists in the inhibition of NR activity by neighbouring inhibitory sequences close to ER bingind sites, so-called inhibitory XREs (Hockings et al., 2006;

Ohtake et al., 2003; Arcaro et al., 1999); the last one, on the other hand, occurs when EDCs interfere with all the processes (biosynthesis, transport to the target tissue and catabolism) on which the hormone availability depends (Baker et al., 1998; You et al., 2001; Boas et al., 2006).

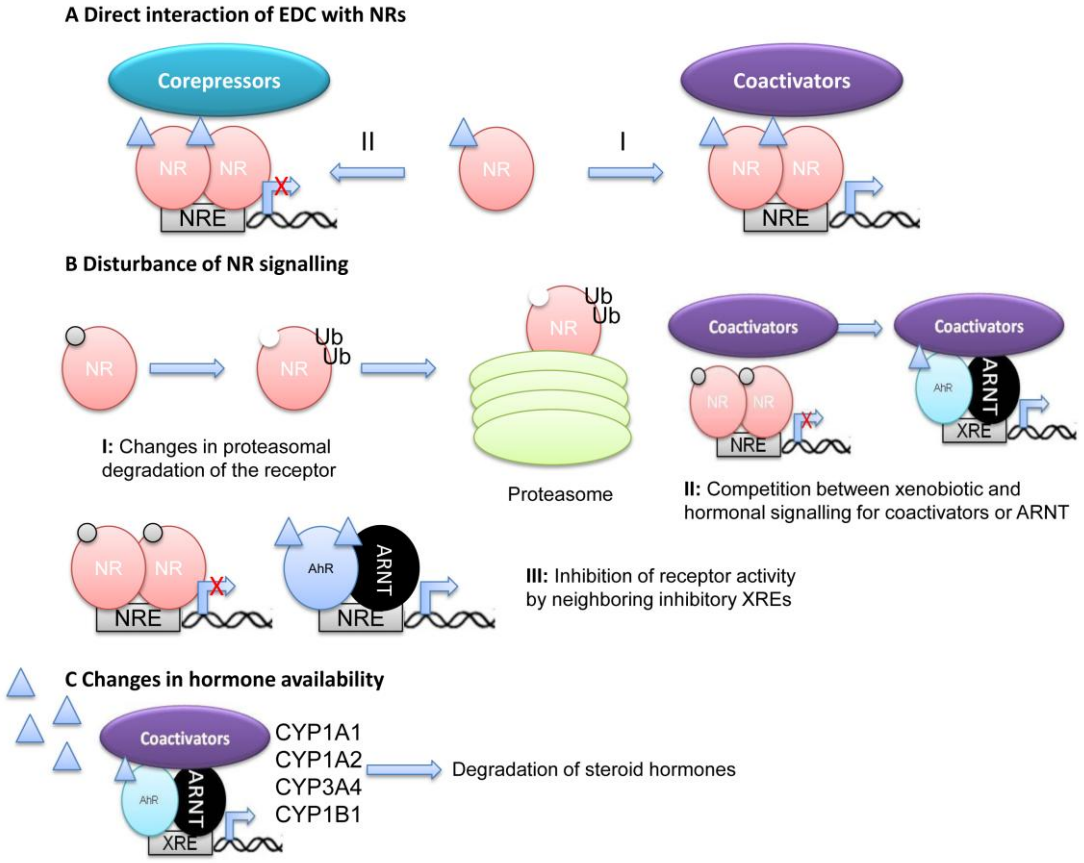


Fig. 8 Mechanisms of endocrine disruption. (A) Many endocrine disruptive chemicals (EDCs) can either act as agonists and induce gene expression (I) or function as antagonists and inhibit the activity of the receptor (II). (B) EDCs can affect receptor function by I: inducing receptor degradation, II: inducing e.g., the AhR signaling pathway, which in turn sequesters common co-activators and ARNT away from the NRs, and III: by associating with the AhR that can bind to inhibitory XREs close to NREs. (C) Enzymes induced by activated AhR are not only involved in metabolism of xenobiotics but also in the catabolism of e.g., steroid hormones. Thus, induction of these enzymes can lead to reduced availability of endogenous hormones.

As a result, the response cascade of natural hormones can either be inhibited or excessively enhanced, at the wrong time, in the wrong tissue (Swedenborg et al., 2009).

These pesticides, such as DDT, not only interfere in estrogen hormone metabolism with mechanisms involving the steroidogenic pathway, receptor mediated

changes in protein levels or antiandrogenic and estrogenic actions (Bradlow et al., 1995; Van Tonder, 2011; Steinmetz et al., 1996) but they also can trigger apoptosis especially through the accumulation of Reactive Oxygen Species (ROS). Interestingly DDT derivates have been shown to induce neural cell death by apoptosis due to the activation of mitogen-activated protein kinases (MAPKs) (Shinomiya and Shinomiya, 2003) and Hexachlorobenzene is known for its capability to directly generate oxidative stress through its property to act as an electron sink (van Ommen and van Bladeren, 1989).

Finally, epigenetic effects have been hypothesized as a possible mechanism of OCPs toxicity. In fact, Shutoh et al. (2009) have shown that DDT affects DNA methylation, that is one main mechanism of epigenetic modulation of genetic expression as suppresses espression of downstream DNA sequences, in experimental animals. Only two epidemiological studies support this hypothesis (Rusiecki et al., 2008; Kim et al., 2010), thus further researches are needed to better understand the relationship between OCPs' exposure and DNA methylation levels in a human population.

Chapter 2

4. BIOACCUMULATION AND BIOMAGNIFICATION

PCBs, as well as dioxins, tends to concentrate in lipid-rich tissues, because of their well-known physicochemical properties, which primarily contribute to their environmental persistence, biomagnification within food chains and, finally, to bioaccumulation, which occurs in several animal species and, clearly, in humans (Burreau et al., 2006). These pollutants reach elevated concentrations both in abiotic and biotic matrices of ecosystems; in fact they have been detected in the air and in the soil and in several terrestrial or aquatic organisms, including humans (Armitage and Gobas, 2007).

Several studies have highlighted the relevance of biological factors (e.g. lipid content, habitat, feeding strategy, trophic level and seasonality) for the POP dynamics in benthic invertebrates (Vieweg et al., 2012).

The elimination of PCBs from aquatic organisms is both species- and congener-specific. Generally, *meta*- and *para*-substituted congeners are easily metabolized (Pruell et al., 1993). The biotransformation of PCBs in vertebrates, as stated in Mechanism of Action paragraph, is mediated by the CYP-dependent mixed-function oxygenase (MFO) (Safe et al., 1985). MFO activity is species-dependent and generally much lower in mussels than in crabs and fish (Porte and Albaiges, 1993). The PCB congeners 110, 138, 149, 153, and 187 are the most aboundant in mussels. The most stable PCB congeners are 138, 153, 170, and 180 in crabs; 138, 153, 170, 180, and 187 in mullet; and 84, 110, 118, and 138 in tuna (Porte and Albaiges, 1993).

Bivalves are widely accepted as sentinels of chemical contamination in seawater because they filter great volumes of seawater, incorporating dissolved pollutants and those associated to the particulate material in their tissues (Léon et al., 2013). Since their low

levels of enzymes responsible for the pollutant metabolism, contaminant concentrations in bivalve edible tissues reflect the magnitude of environmental contamination, providing a time indication of environmental pollution (Otchere, 2005).

The bioaccumulation of pollutants in these organisms is capable of withstanding diurnal fluctuations in the water masses, simplifying the analysis of pollutants due to the higher retention pollution levels attained by the organisms when compared with water or sediment levels (Andral et al., 2004). To date mussels, *Mytilus galloprovincialis*, are commonly used as bioindicators in biomonitoring studies.

Nevertheless the habitat of the analyzed species plays a crucial role in determining the bioaccumulation of PCBs in aquatic animals, in fact, their levels are 500 times higher in the surface strata than in deep water. Consequently, bioaccumulation is several times higher in this zone (Soedergren et al., 1990).

In general, organisms from low trophic levels exhibit relatively lower POP concentrations, which are mostly influenced by their concentrations in seawater (dissolved fraction) and food (e.g. phytoplankton), as well as by the removal/excretion rate from their bodies. Elimination rate of PCB from the tissues is slow, because these contaminants tend to remain stored in lipids. Therefore, greater bioaccumulation occurs in adipose tissue than in muscles or other organs of aquatic organisms (US EPA, 1980). Indeed the aforesaid phoenomen can be argued also to depend on biomagnification.

With this term we indicate a process during which the concentration of a substance increases at each step of the food chain: in a predator the xenobiotic concentration (normalized on the lipid content) is higher than that detectable in the prey. Interestingly, the latter process is responsible for the pollutant persistence in the different matrices of environment, reaching the highest concentrations in tissues of species at top

levels. Therefore, biomagnification is a source of toxicological hazard both for animals and human, which are at the top levels of the food pyramide.

PCBs generally biomagnify within aquatic food chains, as indicated by the PCB concentrations detected in higher trophic levels of aquatic organisms (LeBlanc, 1995; Wilson et al., 1995). This biomagnification is evident in shellfish that accumulate PCBs from consumption of phytoplankton and zooplankton (Secor et al., 1993) and in marine mammals (seals, dolphins, and whales) that accumulate PCBs from consumption of plankton and fish (Andersson et al., 1988; Kuehl et al., 1994; Kuehl and Haebler, 1995; Lake et al., 1995; Salata et al., 1995). Food-chain biomagnification also occurs in several species of fish-consuming birds (Mackay, 1989).

Concentrations of PCBs in common (*Sterna hirundo*) and Forster's terns (*S. forsteri*) (which are primarily fish-feeding birds) are higher than concentrations in tree swallows (*Tachycineta bicolor*) and red-winged blackbirds (*Agelaius phoeniceus*) (which are worm-eating birds) (Ankley et al., 1993). PCB biomagnification in the aquatic food chain is also congener specific (Koslowski et al., 1994). For example, whilst the PCB 138 concentrations increases from plankton (1 µg/kg) to predator fishes (1388 µg/kg in *Morone chrysops*) to herring gulls (*Larus argentatus*) (30063 µg/kg), the congeners 77, 126, and 169 showed no obvious biomagnification in these species. Regarding the PCB 77, the lack of biomagnification has been attributed to its quick elimination by aquatic species (Koslowski et al., 1994).

The biomagnification of PCBs occurs also in the terrestrial food-chain (Hebert et al., 1994). Human exposure to PCBs is mainly due to the dietary intake of contaminated foods, particularly those of animal origin. As demonstrated by Fattore and co-workers (2008), in fact, the occupational exposure rarely occurs, but the human exposure is caused

in the 90 % of examined cases through the intake of contaminated foods (which are of animal origin, mostly ichthiyc and dairy products).

Generally, regarding the assessment of overall PCB contamination levels, we can use as reference value the total concentration of the seven iPCBs (IUPAC nos. 28, 52, 101, 118, 138, 153, 180). These pollutants biomagnify in human diet and are the most abundant congeners in both biotic and abiotic matrices (Storelli and Perrone, 2010). The sum of six iPCBs (except the PCB 118, which is a DL-congener) accounts for the 50 % of the NDL-PCB total amount in the contaminated foods (EFSA, 2005). Therefore, although the NDL-PCBs have been long considered less toxic than DL-congeners, they are equally dangerous since they have been detected at very higher concentration levels in both hematic and tissue samples from exposed subjects (Corsolini et al., 1995).

Concerning the biomonitoring studies conducted in Italy, although it is twenty years since several rules of ban have been issued, elevated levels of PCBs are still found, suggesting that to date these compounds are in the environment also because of the presence of new and continuous emission sources.

It is noteworthy that, regarding the Campania region, high levels of PCB poisoning have been revealed in several species of fish (Ferrante et al., 2007; Naso et al., 2005) and in various species of birds from the coastal areas, suggesting a potential risk for the health of exposed animals and for the ichthiyc products' final consumer.

Fish and dairy products' dietary intake is considered the most important parameter to evaluate in order to perform the risk assessment for the health of the final consumer. In fact, lactation animals and fish, which have been exposed to the aforesaid pollutants through the contaminated environment and/or contaminated foods' intake (cattle feed or preys), concentrate, better than other animals, these compounds. Moreover, during the last

decades, in Campania region, the fish-consumption is increased since fish products are the foremost foods in the Mediterranean diet.

Obviously, also OCPs are responsible for bioaccumulation and biomagnification, since as well as PCBs and PAHs are amongst the main contaminants in the marine environment, with different degrees of resistance to degradation and persistence, and tend to accumulate in marine organisms due to their high hydrophobicity. In fact several studies have highlighted their marked presence in marine organisms and in seawater from Mediterranean area (Montuori et al., 2013; Storelli et al., 2009; Ferrante et al., 2007).

5. TOXICITY

Although most of PCBs doesn't show an acute toxicity but, more frequently, their chemical inertness, remarkable stability and lipophilicity, make them mainly responsible for chronic toxic effects, following their tendency to bioaccumulate in lipid-rich tissue (Table 4).

Table 4. Human health effects associated with exposure to PCBs and other POPs.

Toxicity	Effects	Pollutants	References
NEUROTOXICITY	Decreased scores on the Intelligence Quotient assessment	PCBs and dichloro diphenylethene	Gladen *et al.*, 1988 Lai *et al.*, 1994
	Memory deficits and learning deficits	PCBs and Dioxins	Jacobson and Jacobson, 1996 Vreugdenhil *et al.*, 2004 Patandin *et al.*, 1999
	Lower scores on neurobehavioral tests	PCBs Dioxins	Stewart *et al.*, 2000 Koopman-Esseboom *et al.*, 1996
		PCBs	
ENDOCRINE DISRUPTION	Increased risk of "testicular dysgenesis syndrome"	PCBs	Andersen *et al.*, 2008 Brucker-Davis *et al.*, 2008
	Alteration of timing of menarche	PCBs, HCB and dichloro diphenylethene	Denham *et al.*, 2005
	Changes in thyroid-stimulating hormone levels	PCBs	Chevrier *at al.*, 2007
	Increased obesity development	PCBs	Tang-Péronard *et al.*, 2014
	Onset of type-2 diabetes mellitus and insulin resistance	PCBs and OCPs	Kim *et al.*, 2014
IMMUNOTOXICITY	Thymic involution	PCBs	Park *et al.*, 2008
	Reduced number of NK cells	PCBs 126 and 118	Svensson *et al.*, 1994
	Increased incidence of respiratory and ear infections	PCBs	Weiglas-Kuperus *et al.*, 2004 Dallaire *et al.*, 2006 Dewailly *et al.*, 2000

Health risks due to PCB exposure have been generally assessed on TCDD equivalents (TEQs) using toxic equivalent factors (TEFs) in accordance to the TEF approach firstly adopted by the U.S. EPA (Barnes et al., 1991). The TEF approach has been validated for estimating the risks of non-*ortho*-subsituted coplanar PCBs, which present a toxicological profile similar to dioxin, but it isn't useful for considering adverse effects of *ortho*-subsituted PCBs, which don't possess a dioxin-like toxicity, whilst more frequently elicit effects such as neurotoxicity, immunotoxicity and endocrine disruption.

In animal and human, chronic exposure to PCBs causes several toxic effects, including reduction of body weight, oedema, chloracne, liver hypertrophy, porphyria, estrogen activity, immune suppression and neurotoxicity (Robertson and Hansen, 2001).

Several epidemiological studies have assessed the carcinogenic activity of PCBs and highlighted the correlation between the chronic exposure to PCBs and incidence's increase of cancer of liver, biliary tract and bowel as well as melanoma (Faroon et al., 2001). In the aftermath of Yusho accident, however, there was a statistically significant increase in mortality due to liver cancer in men (but not in women) exposed to PCBs (Kuratsume et al., 1996). Silberhon et al. (1990) have shown that long-term exposure to PCB mixtures in animals is associated with the induction of neoplastic nodules in the liver and, in some cases, of hepatocellular carcinoma if administered in appropriate doses and for long periods.

Previous findings, moreover, demonstrated that halogenated biphenyl and individual congener mixtures, although with different potency, promote carcinogenesis in different *in vivo* models of liver cancer (Glauert et al., 2001). Generally PCBs have a greater carcinogenic activity if they induce the CYP450 (this is possible for PCBs with the higher degree of chlorination). Several studies were carried out also to establish a

correlation between PCB exposure and the incidence of breast cancer. A toxicity assessment analysis conducted in United States has established that no increase of incidence of breast cancer occurs in women with high haematic levels of PCBs. This finding was also confirmed by other studies, which were mainly American, where also the individual congeners were examined (Laden et al., 2001).

In an epidemiological study, conducted in Canada, on the other hand, evaluating the toxicity of individual congeners, it is reported that high levels of PCBs 118 and 156 are associated with an increased risk of breast cancer (Demers et al., 2002).

In fact arguably haematic levels of PCBs should not be taken as a reference, but rather their concentration in adipose tissue, where they tend to accumulate, is a suitable marker of correlation between the exposure to these compounds and the incidence of breast cancer (Archibeque-Engle et al., 1997). Although a subsequent study, carried out in Denmark, has not shown an increased risk for breast cancer in women who have high levels of PCBs in adipose tissue (Raaschou-Nielsen et al., 2005), numerous studies have demonstrated otherwise.

In particular, a study conducted in Spain has shown a strong correlation between PCB 183 levels in breast adipose tissue and the incidence of breast cancer (Lucena et al., 2001). Furthermore Muscat et al. (2003) have identified, performing an analysis of the levels of PCBs in the mammary fat obtained by biopsy, a correlation between their concentration and the incidence of breast cancer characterized by an inauspicious prognosis.

Eum et al., (2004), on the other hand, have proposed the involvement of the Vascular Endothelial Growth Factor (VEGF) in the mechanism of action at the base of carcinogenesis, especially in the case of breast cancer, for the PCB 104. This factor is

known to increase endothelial permeability favouring transendothelial migration of tumor cells.

Conversely, a clinical study about breast cancer, performing a dosage of levels of PCBs in adipose tissue, has showed a protective effect of PCB 156, with anti-estrogenic activity, and a positive association between the risk of this pathology and the levels of congeners 180 and 183 (Holford et al., 2000).

A significant correlation between the PCB 180 levels and the increase of incidence of prostate malignancy was also demonstrated (Ritchie et al., 2003). In this case, the congeners show a structure-dependent profile of activity: PCB 126 and 77, non-*ortho*-substituted congeners, show an anti-androgenic activity mediated by the AhR activation, inhibiting thus the growth of cancer cells; while PCB 118 and 153, *ortho*-substituted congeners, since they do not activate the AhR, stimulate the growth of cancer cells (Endo et al., 2003). In literature only few studies about the relationship between this disease and PCBs exposure are available.

Regarding the colon cancer, in a study conducted in Spain, a clear correlation between the increase of the risk factor (up to three fold) and elevated blood levels of PCBs 28 and 118, mono-*ortho*-substituted congeners, was reported (Howsam et al., 2004). The di-*ortho*-substituted congeners, such as PCBs 101, 153 or 180, on the other hand, do not appear to be involved in the progression of colon cancer. Therefore, from the analysis of this study we can conclude that only mono-*ortho* substituted PCB exposure contribute to the incidence of this pathology. Elevated serum levels of PCBs, as shown by a study conducted in fishermen from the Baltic Sea (Svensson et al., 1995), determine, in addition, a significant increase in the incidence of stomach cancer. Although these subjects presented also significant levels of dioxins and furans, it is plausible to assume that PCBs

pollution also plays a role in the increased mortality due to this pathology. Finally, as regard melanoma, Sinks et al. (1992) have, in a study conducted in workers exposed to PCBs, highlighted an increase in mortality, although no chronic exposure was revealed.

Consistently with this data, a study conducted on a large population of electrical power plant workers from U.S.A. allows us to assert with confidence that following exposure to high levels of PCBs an increase in mortality due to melanoma can be detected (Loomis et al., 1997).

5.1 Immunotoxicity

Few classes of environmental xenobiotics have been extensively studied because of their immunotoxic effects, as well as the halogenated hydrocarbon compounds. They include, in addition to PCBs, polybrominated biphenyls (PBBs), PCDF and dioxins, which target the immune system as their toxicity main organ.

Regarding their immunotoxic effects, various experimental investigations showed, as a result of exposure to these pollutants, immune suppression, with thymic hypoplasia, involution of spleen and lymph node associated tissues, pancytopenia, cachexia and oncogenic promotion (Kramer et al., 2012; Chang et al., 1982; Dewailly et al., 2000; Cocco et al., 2008).

Several comparative studies have shown that animals exposed to PCBs and/or dioxins exhibit immunotoxic reactions very similar to each other. Concerning the sensitivity to the toxicity, many differences in relation to age and sex are found, probably due to the Ah receptor gene polymorphism (Tryphonas, 1994).

DL-PCBs exert strong immunosuppressive effects through a mechanism similar to dioxin, binding thus to the AhR (Kerkvliet, 2009). Although the AhR dependent

mechanism is the best characterized, recent researches have also demonstrated that such immunotoxic effects of NDL-PCBs occur independently of AhR (Duffy and Zelikoff, 2006; Ferrante et al., 2011; Levin et al., 2005a; Lyche et al., 2004). Several indirect mechanisms have been proposed, such as the interference with the signalling via the neural-immune axis, perhaps by affecting calcium homeostasis or serotonergic systems (Duffy-Whritenour et al., 2010; Pessah et al., 2010).

Studies on the resistance or susceptibility to the infections indicate a weakening of immune defences, occurring after a long-term PCB exposure, as demonstrated by the increased susceptibility to *Flavobacterium psychrophilum* infection in rainbow trout *Oncorhynchus mykiss* eggs (Ekman et al., 2004) or to *Staphylococcus aureus* or *Brucella pinnipedialis* hooded seal strain in mice (Imanishi et al., 1984; Nymo et al., 2014).

In vivo studies in rodents and nonhuman primates exposed to PCBs and dioxins have observed the suppression of the antibody response to immunization with sheep red blood cells and the reduction of the response to mitogen in lymphocyte proliferation assays, providing thus a clear body of evidence for the immunosuppressive effects of Aroclors and DL-PCBs (Kramer et al., 2012).

Bone marrow hypocellularity and atrophy of lymphoid organs, including the thymus and spleen, have been observed in several animal species following oral, dermal, and transplacental exposure to high- and low-dose PCB mixtures and certain congeners (Kimbrough et al., 1978; National Toxicology Program (NTP), 2006; Smialowicz et al., 1989).

Consistent with these findings, a clinical study examining thymus growth after PCB exposure found that among a cohort of 982 mother–infant pairs from a PCB-contaminated region in Eastern Slovakia, prenatal PCB exposure, assessed through

maternal serum PCB levels, was associated with significantly reduced thymus volume at birth (Park et al., 2008).

Human studies have provided evidence supporting the effects of PCBs on several markers of normal immunosurveillance and tumor control. Among these markers, natual killer (NK) cells are particularly important in cancer immunosurveillance because of their ability to eliminate early malignant cells without activation by $CD4^+$ cells. A study of heavy fatty-fish consumers and non consumers in Sweden (Svensson et al., 1994) found reduced levels of NK cells in the peripheral blood of fatty-fish consumers and a significant negative correlation between weekly fish intake and proportions of NK cells. In a subsample of 11 participants, significant negative correlations were observed between number of NK cells and blood levels of PCBs 126 and 118.

Victims of the Yu-Cheng PCB/PCDF poisoning were found to have fewer monocytes and polymorphonuclear leukocytes (PMNs) bearing immunoglobulin and complement receptors (Chang et al., 1982), and among 146 workers occupationally exposed to PCBs for a minimum of 6 months, blood levels of PCB 138 over the mean of healthy controls were associated with a greater frequency of undetectable levels of interleukin (IL)-4 (Daniel et al., 2001).

To date all researchers agree in defining the perinatal period as the time of greater sensitivity to the immunotoxicity of these environmental pollutants. PCB exposure, as estimated by various measures including PCB levels in cord blood, maternal sera, and breast milk, has been associated with an increased incidence of respiratory infections, ear infections, influenza, and chicken pox in healthy Dutch (Weisglas-Kuperus et al., 2000; 2004) and Inuit (Dallaire et al., 2006; Dewailly et al., 2000) preschoolers; children of capacitor manufacturing workers, particularly those breast-fed for lengthy periods (Hara,

1985); and children exposed to PCBs and PCDFs during the Yusho and Yu-Cheng poisoning incidents (Chao et al., 1997; Guo et al., 2004; Yu et al., 1998).

PCB exposure has also been associated with insufficient response to vaccination. In two birth cohorts from the Faroe Islands with pre- and post-natal PCB exposure from dietary consumption of whale blubber, insufficient antibody response to diphtheria and tetanus toxoids was associated with PCB exposure (Heilmann et al., 2006; 2010).

Epidemiological studies show that PCBs are associated with modification of both innate and adaptive immunity, including effects on immune cells and signalling molecules, with implications for both immune response and initiation. Such effects are manifested as an increased incidence of infections, insufficient antibody response to vaccination, and changes in immune organs, lymphocyte subsets, and lymphocyte function.

Studies in both adults and children have demonstrated an association between PCB exposure and reduced functional responsiveness of lymphocytes, as indicated by decreased responses to mitogen stimulation (Belles-Isles et al., 2002; Bilrha et al., 2003; Daniel et al., 2001; Nakanishi et al., 1985) and decreased delayed-type hypersensitivity reactions (Lu and Wu, 1985; Van Den Heuvel et al., 2002). Additionally, studies have demonstrated correlations between PCB exposure or markers of PCB exposure and alterations to normal lymphocyte subpopulations. Hagmar et al. (1995) observed dose-related inverse associations between fatty fish consumption, an important source of exposure to PCBs and other POPs in certain areas of the world, and the proportion of cytotoxic T cells in Latvian villagers. Daniel et al. (2001) found that a greater proportion of individuals with elevated blood levels of PCB 101 had low DR^+ cell counts (a measure of antigen-presenting cells) among 146 workers occupationally exposed to PCBs for a minimum of 6 months. Lawton et al. (1985) studied 194 capacitor workers exposed to

Aroclors (industrial mixtures of PCBs) before and after the factory discontinued use of PCBs and found abnormally high lymphocyte counts among workers before PCB discontinuation.

Reductions in these early innate immune response cells, including monocytes, PMNs, and NK cells, can impair the ability of the immune system to identify foreign antigens and limit the early cytokine and chemokine signalling that initiates a robust and complete immune response (Murphy et al., 2008). T cells also play a role in cancer immunosurveillance, and PCBs have been shown to affect T-cell number, function, and maturation (Belles-Isles et al., 2002; Bilrha et al., 2003; Weisglas-Kuperus et al., 2000).

Though toxicological studies on NDL-PCBs are in their infancy, our recent *in vitro* data demonstrate the ability of NDL-PCBs to suppress the macrophage response and support an apoptotic mechanism initiated by PCBs 101, 153, and 180 (Ferrante et al., 2011). Additionally, Levin et al. (2005a) showed that exposure of healthy human leukocytes to the NDL-PCBs 138, 153, and 180 resulted in reduced phagocytosis mediated through both reduced neutrophil and monocyte activity, toxic effects were more pronounced after exposure to mixtures of multiple NDL-PCBs. In contrast, exposure to the DL-PCB 169 or to TCDD did not affect phagocytosis, suggesting an independent immunotoxic effect of NDL-PCBs.

Conversely, the toxicological literature demonstrates strong evidence for an effect of PCBs on inflammation (Strauss and Heiger-Bernays, 2012). Mechanistic studies indicate that DL-PCBs target the acute phase of inflammation (Hennig et al., 2002; Kerkvliet, 2009), but no studies are available regarding the impairment of immune innate response due to NDL-PCB exposure.

5.1.1 LPS-activated macrophages

Normally, macrophages recognize patterns of foreign biological substances through the activation of TLRs that initiate various signalling pathways leading to production of proinflammatory cytokines, such as IL-6 and TNF α.

LPS is the major molecular component of the outer membrane of Gram-negative bacteria and a potent natural immune stimulator. Exposure to LPS has been shown to cause a wide range of acute physiological and cytotoxic effects in a variety of cells (Karahashi and Amano, 2003). Microorganism-associated molecular patterns are recognized by pattern-recognition receptors such as TLRs, and, in particular, LPS was identified as a ligand of TLR-4.

TLR-4 is the receptor for bacterial endotoxins (LPS) both in mouse and in human cells, and activation of TLR-4 by LPS initiates innate immunity and inflammation (Peri et al., 2012). One of the critical events in TLR-4 inflammatory response is the increased expression of various cytokines (Rodriguez-Vita and Lawrence, 2010). These molecules are recognized as a sign of bacterial infection, responsible for the development of local inflammatory response and, in extreme cases, septic shock. After the binding of the endotoxin to the TLR-4, which is also mediated by the coreceptor CD14, a downstream activation of NF-κB or IRF3, via the adaptors MyD88 and TRIF, occurs, inducing the transcription of cytokine and chemokine genes that collectively ramp up the host's immune defense mechanisms (Fig. 9).

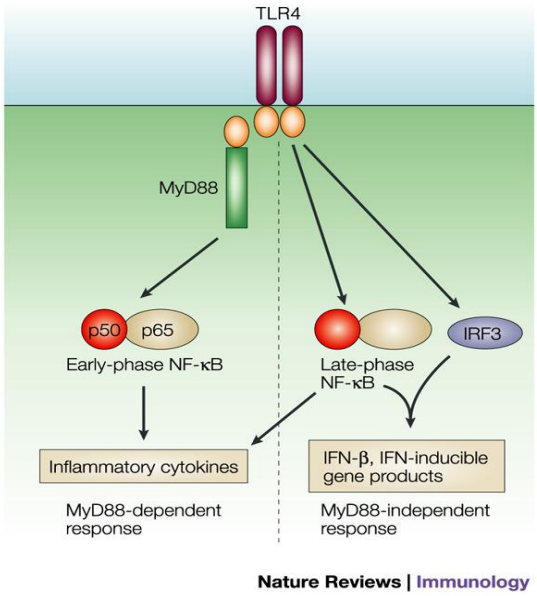


Fig. 9 Stimulation of Toll-like receptor 4 (TLR4) facilitates the activation of two pathways: the myeloid differentiation primary-response protein (MyD) 88-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of nuclear factor (NF) -kB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) and involves the late phase of NF-kB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes.

5.1.2 IL-6

IL-6 is a pleiotropic cytokine with various biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis (Kimura and Kishimoto, 2010). IL-6 is secreted by many cell types, including immune cells, fibroblasts, endothelial cells, myocytes, and a variety of endocrine cells (Fried et al., 1998).

It is a multifunctional cytokine that has been well known for its anti-inflammatory and/or proinflammatory effects in immune responses. IL-6 signals through a complex consisting of its receptor IL-6R and signal-transducing component glycoprotein (gp) 130, leading thus to the activation of the receptor IL-6R. IL-6R exists in both a transmembrane and a soluble form. IL-6 binds to both these forms, which can then interact with gp130 to trigger downstream signal transduction and gene expression. Although gp130 has no

intrinsic kinase domain, members of the Janus Kinase (Jak) family, such as Jak1, Jak2, and tyrosine kinase 2 (Tyk2), are constitutively associated with gp130. Complexes of IL-6, IL-6R, and gp130 phosphorylate these kinases and then activate the cytoplasmic transcriptional factors, STAT1 and STAT3 (Stahl et al., 1994). Thus, IL-6 activates these kinases and transcriptional factors through IL-6R/gp130 complexes, which in turn leads to IL-6's downstream effects.

Recently it has been demonstrated that IL-6 plays a crucial role in regulating the balance between IL-17-producing Th17 cells and regulatory T cells (Treg) (Kimura and Kishimoto, 2010). The two $CD4^+$ T-cell subsets control the immune functions: Th17 cells are involved in the pathogenesis of autoimmune diseases and protection against bacterial infections, while Treg block excessive effector T-cell responses. IL-6 induces the development of Th17 cells from naive T cells together with TGF-β; in contrast, IL-6 inhibits TGF-β induced Treg differentiation. Deregulation or overproduction of IL-6 leads to autoimmune diseases and a reduced defences against bacterial infections.

5.1.3 TNF-α

TNF-α is a pro-inflammatory cytokine, secreted mostly by activated macrophages and lymphocytes, which plays a key role in the acute phase of inflammatory responses.

Hong et al. (2004), for the first time, have studied the effects of several endocrine disrupting chemicals (EDCs), which resemble to and include PCBs, on LPS-induced TNF-α production by mouse macrophages. They have shown that these compounds can both induce or suppress the LPS-induced TNF-α production, suggesting that they may affect the development of infectious diseases. In particular, they could be harmful also ihnibiting the

endotoxin-induced release of this mediator, because this cytokine plays an important role in bacterial clearance.

5.1.4 MCP-1

MCP-1 (or CCL2), a member of the chemokine (C-C motif) subfamily, is a potent mononuclear cell chemoattractant produced by different cell types including macrophages, monocytes and epithelial cells in response to oxidizing agents, cytokines, growth factors and endotoxins (Yadav et al., 2010). Although MCP-1 is constitutively produced, higher concentrations are observed during the inflammatory response. MCP-1 controls the monocyte/macrophage phenotype profile and monocyte traffic during inflammation by interacting with G-protein-coupled receptors; chemokine (C-C motif) receptor 2 and the Duffy antigen receptor for chemokines (DARC) are expressed on leukocyte membranes (Deshmane et al., 2009; Yadav et al., 2010).

Besides the LPS-stimulated cytokines, such as TNF-α, IL-1β, IL-6, also MCP-1 is also known to trigger and induce TLR-4 downstream signal transduction pathways and to promote immune responses.

5.1.5 COX-2 and iNOS expression by LPS trhough NF-kB activation in immunocompetent cells

Immunocompetent cells, such as macrophages, have a wide repertoire of chemical signals to communicate with the other cells. For example, macrophages stimulated by LPS generate excess nitric oxide (NO) through the action of iNOS, a feature of classically activated macrophages. NO has been involved in the earlier phases of innate immune response to bacterial infection. This function plays a significant role in microorganism

clearance, anyway NO can also have detrimental effects on the host by generating oxidative or nitrosative stress. In fact during inflammation, such as the process of host defence, endotoxins and cytokines induce rapid alterations in cellular immediate-early gene expression, leading to the *de novo* synthesis of COX-2 (Di Rosa et al., 1996; Mitchell et al., 1994) and iNOS (Meli et al., 2000). Co-induction of iNOS and COX-2 has been shown in several cell types, including murine macrophages (Akarasereenont et al., 1994; Salvemini et al., 1993; Swierkosz et al., 1995) with similarities in the signal transduction pathways.

Differently from their respective constitutive enzymes (COX-1 and eNOS), the inducible isoforms are responsible for the production of large amounts of NO and prostaglandins (PGs) at the inflammatory site (Lee et al., 1992; Nussler et al., 1993).

Several studies report that the expression of a variety of chemotactic and inflammatory cytokines, cytokine receptors, and inducible enzymes, such as COX-2 and iNOS, involved in the synthesis of pro-inflammatory mediators, are regulated by NF-kB pathway (D'Acquisto et al., 1997; Kleinert et al., 2003).

NF-kB activation (Fig. 10) is regulated by a related inhibitory protein called IkBα which retains NF-kB in the cytoplasm. In response to a proinflammatory signal, IkBα is rapidly phosphorylated, polyubiquitinated and degradated by the 26S proteasome. Thus NF-kB enters into the nucleus to direct transcription of specific pro-inflammatory genes (Baeuerle and Baltimore, 1996; Thanos and Maniatis, 1995).

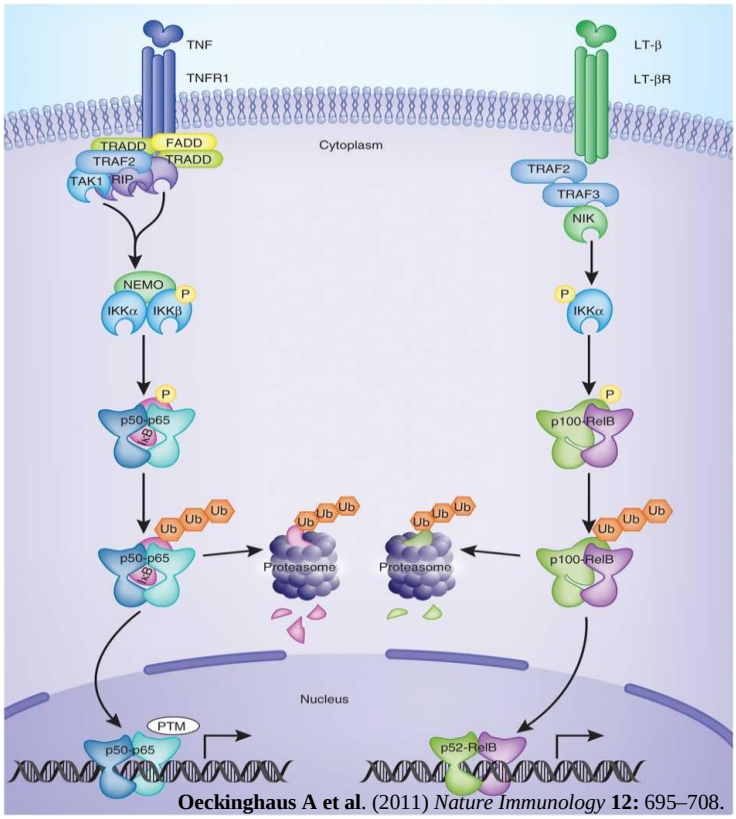


Fig. 10 Under resting conditions, NF-κB dimers are bound to inhibitory IκB proteins, which sequester inactive NF-κB complexes in the cytoplasm. Stimulus-induced degradation of IκB proteins is initiated through phosphorylation by the IκB kinase (IKK) complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Phosphorylated IκB proteins are targeted for ubiquitination and proteasomal degradation, which thus releases the bound NF-κB dimers so they can translocate to the nucleus. NF-κB signaling is often divided into two types of pathways. The canonical pathway (left) is induced by most physiological NF-κB stimuli and is represented here by TNFR1 signaling. In contrast, the noncanonical pathway is induced by certain TNF family cytokines, such as CD40L, BAFF and lymphotoxin-β (LT-β).

5.2 PCB exposure and endocrine disruption

The term "endocrine disrupter (ED)" was first used at the Wingspread Conference in 1991, and referred to those endocrine active substances which may lead to an adverse health effect (Colburn and Clement, 1992). These chemicals are able to disrupt the closed feedback loops of the hormonal and homeostatic systems (Diamanti-Kandarakis et al., 2009). The group of known EDs is extremely heterogeneous. It embraces ubiquitous synthetic substances used as industrial lubricants and solvents, and their by-products: PCBs

(Pocar et al., 2012), polybrominated diphenyl ethers (PBDEs) and dioxins such as TCDD; plastics: bisphenol A (BPA) and bisphenol S (BPS); plasticisers: phthalates; pesticides: atrazine, cypermethrin, DDT, dieldrin, methoxychlor (MXC) and vinclozolin (VCZ); and drugs: diethylstilbestrol (DES) and ethinyl oestradiol (EE), as well as non-steroidal anti-inflammatory drugs (NSAID) and acetaminophen. Natural chemicals such as genistein, a phytoestrogen, and heavy metals can also have endocrine-disruptive effects.

All these aforesaid chemicals may mimic or blunt the biological activity of a hormone by binding to the specific cellular receptor, thereby inducing (agonistic effect) or preventing (antagonistic effect) or diminishing the action of the corresponding endogenous hormone.

Some EDs are also capable of modifying hormone bioavailability by interfering with its secretion and transport or disrupting the enzymatic pathways involved in hormone synthesis and metabolism (Phillips et al., 2008; Whitehead et al., 2006).

About the effects of PCBs on endocrine system, several *in vivo* studies have demonstrated that ingestion of these pollutants was associated with adverse reproductive effects in adult experimental animals (including rats, mice, minks, and monkeys) as well as in their offspring. Reproductive effects in females included prolonged estrus, decreased sexual receptivity, decreased implantation rate, decreased conception rate, prolonged menstruation, and decreased fertility (Meerts et al., 2004; Kuriyama and Chahoud, 2004).

Reproductive effects in males included multigenerational effects: the third generation of mice exposed *in utero* to environmental levels of PCB presented morphological reproductive abnormalities and impaired gamete quality (Pocar et al., 2012). Moreover, thyroid hormone regulation has been shown to be affected in rats following prenatal and/or lactational exposure to either mixtures or individual congeners of

PCBs. A decrease in circulating thyroxine (T4) levels has been consistently seen in mothers and their offspring, while effects on triiodothyronine (T3) levels have been more variable and may depend on the type of PCB the animals were exposed (Provost et al., 1999).

Epidemiological data suggest that human developmental exposure to environmental levels of PCBs is indeed connected to an increased risk of "testicular dysgenesis syndrome" features such as hypospadias and cryptorchidism (Andersen et al., 2008; Brucker-Davis et al., 2008; Cai et al., 2011). Furthermore, a recent study has shown that women with endometriosis have strongly higher concentrations of PCBs and TCDD in the peritoneal fluid, possibly leading to chronic inflammation, which may result in the stimulation of endometrial cells derived from retrograde menstruation (Rier, 2008).

Among the above mentioned effects, PCBs also target the adipose tissue (AT), whose metabolic and endocrine functions have been recently recognized.

Recently, various interactions between AT and POPs have been reported, suggesting that this tissue plays a significant role in the kinetics and the toxicity of POPs (Kim et al. 2011, 2012). As reviewed by La Merrill and co-workers (2013) AT has three basic toxicological implications: 1) it acts as a reservoir for a variety of organic xenobiotic chemicals, in particular POPs; 2) it constitutes a low-grade internal source of stored POPs leading to continuous exposure of other tissues; and 3) it can be a target for the POP which can affect AT functions, increase its inflammatory state, and/or modulate the differentiation of adipocyte precursor. As a consequence, targeting this tissue, PCBs can exert an obesogenic effect. The prevalence of obesity was increased dramatically over the last two to three decades and in the western world it is reaching epidemic proportions (Lobstein et al., 2003; Ogden et al., 2014). Overweight and obesity causally contribute to a

number of serious medical conditions, such as cardiovascular diseases, type-2 diabetes and some forms of cancer. Increasing evidence suggests that the commonly held causes of obesity, which are over-eating, inactivity and genetic pre-disposition, do not fully explain the current obesity epidemic. Interestingly, the production and use of synthetic chemicals have increased dramatically, in parallel with growing obesity (Baillie-Hamilton, 2002) and it has been suggested that EDCs may play a key role in obesity development by altering metabolic control mechanisms (Baillie-Hamilton, 2002).

This hypothesis is supported by several *in vitro* and *in vivo* studies, as well as epidemiological evidence, demonstrating that some ED-chemicals (EDCs) (e.g. PCBs) may affect adiposity and obesity incidence. These EDCs, indeed, have been defined as obesogenic compounds (Dirinck et al., 2011).

Recently Chapados et al. (2012) have highlighted an increased proliferative effect on human preadipocytes by OCs, such as the NDL-PCB 153 and the DDE. Conversely, the exposure to PCB 77 had no effect on the proliferative capacity of human preadipocytes. Their findings suggest that acute exposure to some OCs increases the proliferative capacity of human preadipocytes (28–72%). Moreover, previous studies, conducted on several types of cells, have corroborated these results, confirming the proliferative effect of the aforesaid OCs (Radice et al., 2008; Chang et al., 2008; Endo et al., 2003).

The literature is very limited in providing reasons for which some cell lines show dose-dependent responses of OC exposure on proliferation while others do not. This could be partly explained by an on–off response at some threshold OC concentrations. One mechanism proposes the proliferative effects of NDL-PCB 153 to be mediated by the mitogen-activated protein kinase (MAPK) pathway activation of extracellular-regulated kinase 1 and 2 (ERK1/2). The lower affinity of PCB 77 to AhR compared to TCDD may

explain the absence of difference in the proliferative capacity between control and PCB 77 treated preadipocytes.

Therefore some OCs can increase the proliferative capacity of human fat cell precursors, probably promoting a fat mass gain.

The exposure to some EDCs has been also associated with other obesity-related metabolic diseases, such as metabolic syndrome and type 2 diabetes mellitus. Accordingly, this subclass of EDCs that perturbs metabolic signalling has been defined as metabolic disruptors (Casals-Casas et al., 2008). Mechanistic studies suggest that they downregulate nuclear and energy expenditure related receptors, such as the estrogen-related receptor α (ERRα), the peroxisome proliferator-activated receptor (PPAR) α/δ and the PPAR γ coactivator (PGC)-1α, and lipogenesis involved receptors (PPAR γ and sterol regulatory element-binding protein (SREBP)-1c. They also affect the estrogen biosynthesis in WAT, targeting the enzyme aromatase and leading thus to their toxic effects on metabolic functions.

The above experimental and epidemiological findings demonstrate that EDCs, through the interaction with nuclear receptors, not only can be defined as endocrine, but also as metabolic disrupter chemicals.

5.2.1 Adipogenic differentiation

Adipogenesis is a highly controlled process, whose cellular and molecular proceedings have been extensively studied in recent years. It is characterized, in the early stages, by the activation and/or transcriptional repression of genes expressed in preadipocytes (Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000). Firstly, the arrest of growth occurs. Afterwards, preadipocytes need to receive an appropriate

combination of mitogenic and adipogenic signals to continue through the later stages of differentiation, leading to the progressive acquisition of morphological and biochemical characteristics typical of mature adipocytes. Adipogenic differentiation starts through the drastic transition of preadipocytes from the elongated shape of fibroblasts to that spherical typical of adipocytes. The morphological changes are, then, accompanied by changes in the type of components of the extracellular matrix (ECM) and the cytoskeleton (Gregoire et al., 1990).

The terminal stage of differentiation is characterized by the activation of the transcriptional cascade that leads to an increased expression of important proteins involved in triglycerides synthesis, glucose transporters expression and insulin-sensitivity. In this last phase, the newly formed adipocytes have become highly specialized endocrine cells capable of secreting key hormones in regulating the energy homeostasis (Gregoire et al., 1990).

Mature adipocytes are the major cellular components of white adipose tissue (WAT). Under a strict hormonal control, in these cells it has been described a fine regulation of storage/catabolism processes of metabolites, most of which are lipids, on which the energetic balance control depends. However, WAT plays a much more complex and dynamic role, secreting several factors, involved in immunological response, vascular diseases and appetite regulation.

In addition to proteins involved in lipid and lipoprotein metabolism, adipocytes also synthesize cytokines, growth factors and mediators involved in the regulation of food intake and energy homeostasis. These factors may play a crucial role in energy homeostasis and/or control key functions in WAT both in physiological or in pathological conditions.

5.2.2 Leptin

The hormone leptin (Fig. 12), whose nomenclature is derived from the Greek word "λεπτός" (means thin), is a 16 kDa protein that has a primary role in suppressing appetite and increasing energy expenditure through metabolism (Zhang et al., 1994).

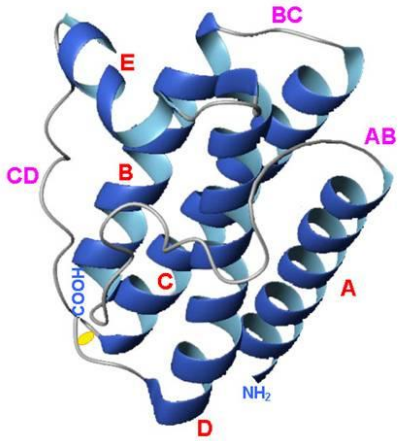


Fig. 12 Three-dimensional structure of leptin.

Leptin is secreted mostly by WAT and, at hypothalamic level, plays a crucial role in regulating food intake, inducing thus satiety. However, leptin is not the only afferent signal that regulates food intake and body weight. In fact, feeding behaviour and energy balance are regulated in short term and long term systems. The short-term system controls feeding behaviour throughout the day. Several studies indicate that variations in the plasma glucose concentrations, body temperature, cholecystokinin and other hormones can rapidly modulate the food intake (Coppola and Diano, 2007). The long term system, on the other hand, controls the balance between food intake and energy expenditure, regulating the amount of energy reserves in the body.

Leptin appears to play an important role in the long-term regulation and affects the amount of ingested food compared with the rate of energy expenditure.

5.2.3 Ob expression

In mice leptin gene (ob) is located on chromosome 6, while in humans on chromosome 7 (Zhang et al., 1994). This 650 kb gene consists of three exons separated by two introns. The region encoding for the leptin is localized in exons 2 and 3. Within the ob gene promoter area have been identified several regulatory elements that include cAMP, glucocorticoid response elements, CCAAT/enhancer binding sites, and SP-1 (Gong et al., 1996; Hwang et al., 1996; Miller et al., 1996).

Interestingly, leptin presents striking structural similarities to members of the long-chain helical cytokine family (Madej et al., 1995; Zhang et al., 1997). The most conserved regions observed between leptin proteins of different species are located within four α-helices (Zhang et al., 1997). A disulphide bond between cysteine 96 and cysteine 146 appears to be important for structure folding and receptor binding because mutations of either cysteine render the protein biologically inactive (Zhang et al., 1997). Both the crystallographic or the NMR analysis revealed that leptin has a tertiary structure resembling that of members of the long-chain helical cytokine family, such as somatotropin (GH), prolactin (PRL), eritropoyetina (EPO) and interleukins (IL-6, IL-11 and IL-12) (Zhang et al., 1997).

Leptin is secreted by adipocytes in relation to triglyceride volume and content (Zhang et al., 2002). WAT ob gene expression and circulating leptin levels are regulated in a short-term fashion. They increase in response to glucocorticoids, insulin, TNF-α, IL-1 and estrogen (Shimizu et al., 1997), but decrease in response to testosterone, the sympathomimetic amines, or exposure to cold and fasting (Rayner and Trayhurn, 2001).

Leptin secretion follows a circadian rythm: increases during the night, peaking in the early hours of the day and, conversely, decreases in the middle of the day (Flier and

Maratos-Flier, 2010). This rhythm was recorded in both healthy and diabetic, lean or obese individuals. The timing of secretion is similar to those of prolactin (PRL), thyrotropin (TSH), melatonin and free fatty acids, while it differs from that of cortisol and adrenocorticotropic hormone (ACTH) (Mantzoros and Moschos, 1998).

Leptin circulates in plasma as a free peptide or associated to the soluble isoform of its receptor (Ob-Re) (Sinha et al., 1996). At blood-brain barrier (BBB) level, there is a saturable transport system, probably constituted by the short isoform of leptin receptor, which facilitates the entry of the hormone in the CNS, where it binds to the long functional isoform of leptin receptor (Ob-Rb) in the hypothalamus, where it acts as a satiety factor (Gavrilova et al., 1997).

Leptin circulating levels, especially in the lean subjects, are positively correlated with the body mass index (BMI) (Sinha et al., 1996). Previously it has been shown an increase in leptin concentrations in cerebrospinal fluid and plasma from obese subjects compared with lean ones; probably because of its less effective transport from cerebrospinal fluid to Central Nervous System (CNS). Thus a relative leptin deficiency in the CNS of obese subjects has been recorded (Schwartz and Seeley, 1997), consistently with the leptin resistance, often associated to the obese phenotype.

5.2.4 Ob-Rb signal transduction pathway

Tartaglia et al. (1995) have identified and cloned the leptin receptor, Ob-R, expressed in the mouse choroid plexus. Ob-R is homologous to members of the cytokine receptor superfamily and its mRNA expression pattern suggests that it may be expressed by various tissues, including the hypothalamus. The family of class I cytokine receptors also includes the receptors for IL-12, IL-6, IL-11, in addition to those for the growth

hormone, for the PRL and for EPO (Tartaglia et al., 1995). Members of this family have conserved extracellular motifs formed by four cysteine residues and a WSXWS motif (Trp-Ser-xaa-Trp-Ser) (Bazan, 1989). Leptin receptor mRNA was localized in the hypothalamus by in situ hybridization, being particularly abundantly expressed in the arcuate nucleus (ARC), such as in the dorsomedial (DMH) and paraventricular nuclei (PVN). Each of these nuclei plays an important role in the regulation of energy balance (Mercer et al., 1996).

Ob-R presents several isoforms, all produced by tissue-specific alternative splicing: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf (Fig. 13; Lee et al., 1996).

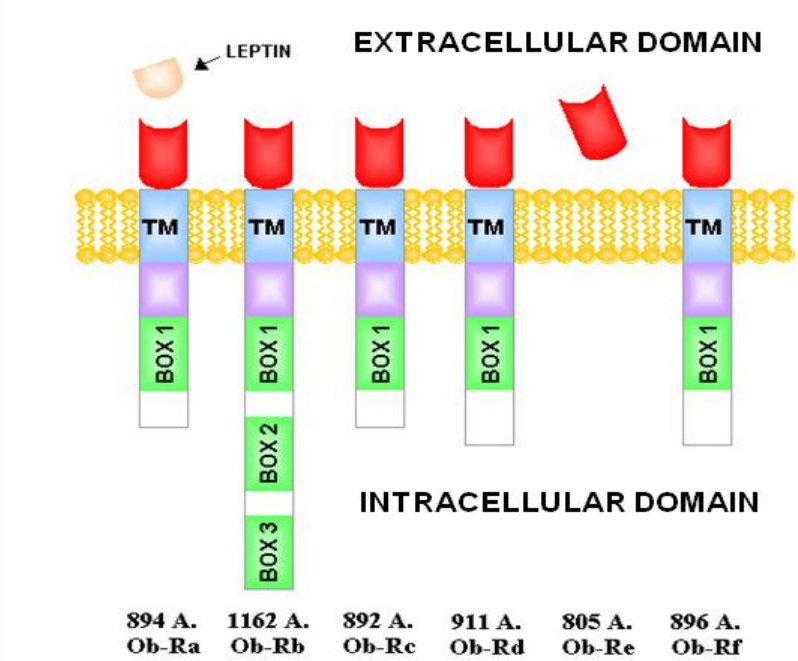


Fig 13 Structure and isoforms of leptin receptor. Ob-Rb contains the longest intracellular domain, which is crucial for leptin signalling. Ob-Ra, Ob-Rc and Ob-Rd contain only short cytoplasmic domains. Ob-Re is a secreted isoform of the leptin receptor, lacking transmembrane and cytoplasmic parts.

The extracellular domain is composed of 820 aminoacids. All isoforms share the same extracellular domain in the amino-terminal portion but differ in the carboxy-terminal portion. Ob-Ra has a short cytoplasmic domain and acts as a leptin transporter through the BBB (Lee et al., 1996; Chen et al., 1996). Conversely, Ob-Re is the unique soluble isoform

of leptin receptor, without the typical transmembrane and intracellular portions (Chen et al., 1996). Ob-Re binds leptin with high affinity, increasing serum levels of the biologically active isoform and delaying its clearance. Leptin is eliminated mainly by glomerular filtration through the kidney and by degradation within renal epithelium cells.

Ob-Rb is physiologically expressed both in hypothalamic neurons and in other cells such as $CD4^+$ T-lymphocytes (Lord et al., 1998), macrophages, cells of the pancreatic islets, vascular endothelial cells and white and brown adipose tissue cells (Lord et al., 1998; Lee et al., 1996; Ghilardi et al., 1996; Sierra-Honigmann et al., 1998).

All these receptors lack enzymatic motifs in their cytoplasmic domains and instead associate with members of the janus kinase (Jak) family, a class of cytoplasmic tyrosine kinases. Moreover, only the long isoform Ob-Rb has an extended cytoplasmic domain and mediates the hormone signal transduction. For this reason, this isoform is responsible of the biological effects of leptin (Dallongeville et al., 1998).

Ob-Rb is the only receptor isoform that can signal intracellularly via the Jak-signal transducer and activator of transcription (STAT) signal transduction pathway (Malendowicz et al., 2006). Binding of ligand to the receptor activates the Jak kinase and leads to phosphorylation of cytoplasmic target proteins. Among these, there are also the intracellular domains of the receptors and a class of cytoplasmic transcription factors called STAT. To date six members of the STAT family have been identified. Phosphorylation of STAT proteins induces dimerization and translocation into the nucleus and results in specific activation of gene transcription. As for other members of the cytokine receptor family, it was suggested that dimerization of the cytoplasmic region of the OB-Rb is required for signal transduction. The formation of its dimer occurs after its interaction with leptin (Devos et al., 1997). Afterwards Ob-R undergoes a conformational change, exposing

the binding domain to the tyrosine kinase JAK2; Ob-Rb, then, recruits JAK2 which mediates subsequent phosphorylations. JAK2 is activated and phosphorylates the receptor on two tyrosine residues: Tyr_{Y985} and Tyr_{Y1138}. STAT3 is known to be on the latter residue, which was previously phosphorylated by JAK2. STAT3 is a transcription factor that, although normally connected to the intracellular domain, once phosphorylated, dimerizes and translocates into the nucleus where activates the expression of several genes including c-fos, c-jun, tis-11 and the suppressor cytokine signalling (SOCS) 3 (Fig. 14).

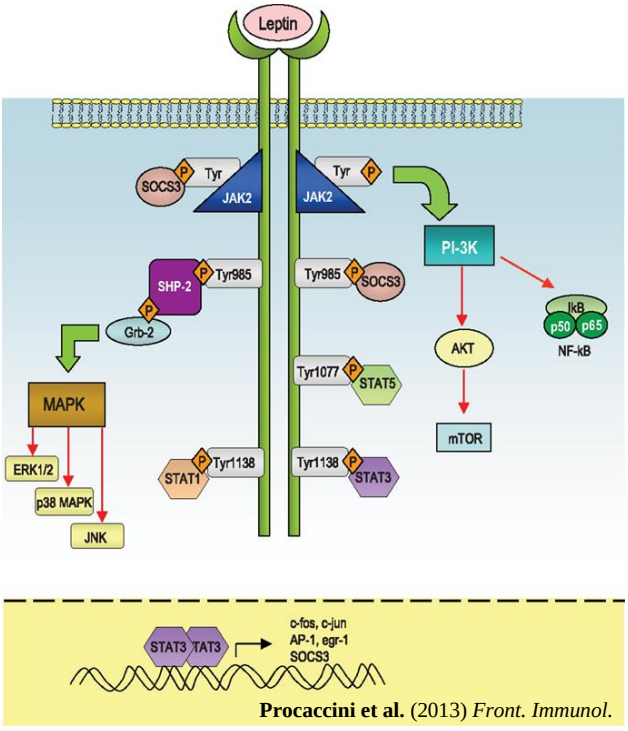


Fig. 14 Schematic representation of the leptin-induced pathways. After leptin binds to the long isoform of the leptin receptor (ObRb), Jak2 is activated at the box 1 motif, resulting in the autophosphorylation of tyrosine residues and phosphorylation of tyrosines that provide docking sites for signaling proteins containing src homology 2 (SH-2) domains. The autophosphorylated Jak2 at the box 1 motif can lead to activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Akt can regulate a wide range of targets including FOXO1 and NF-κB. Both Tyr1077 and Tyr1138 bind to STAT5, whereas only Tyr1138 recruits STAT1 and STAT3. STAT3 proteins form dimers and translocate to the nucleus to induce expression of genes such as c-fos, c-jun, egr-1, activator protein-1 (AP-1), and suppressors of cytokine signaling 3 (SOCS3).

STAT3 has been found in high concentrations in various hypothalamic nuclei, and in other areas of the CNS, suggesting that the central effects of leptin are mediated from STAT3 itself (Hakansson and Meister, 1998; Stromberg et al., 2000). It has been shown that leptin can induce the expression of SOCS3 mRNA in the same regions of the hypothalamus that express Ob-Rb (Bjørbaek et al., 1998). In particular, SOCS3 induced a

reduction of leptin-induced STAT3 phosphorylation through the binding to Tyr_{985} of leptin receptor (Bjørbaek et al., 1999; 2001).

5.2.5 Leptin plays a crucial role in regulating lipid metabolism

Leptin regulates both adiposity and energy homeostasis, through the decrease in food intake and partitioning of metabolic fuels toward utilization and away from storage. This adipokine directly affects lipid metabolism, reduces the biosynthesis of fatty acids and triglycerides and, concomitantly, promotes β-oxidation (Muoio et al., 1997). This latter effect is due to the reduction of acetyl CoA carboxylase (ACC) activity. This is known to be the rate limiting enzyme of fatty acid metabolism and, interestingly, after leptin administration, it has been shown a reduction of its expression (Zhou et al., 1997). Leptin, moreover, increases the mRNA levels of the carnitine acyltransferase I (Zhou et al., 1997), inhibiting thus the byosinthesis of fatty acids and promoting their transfer into mitochondria, where β-oxidation occurs. Shimabukuro et al. (1997) have demonstrated that leptin stimulates the oxidation of fatty acids, probably through the induction of the synthesis of oxidative enzymes, and is responsible of a reduction of the fat mass. Conversely, low circulating levels of the hormone, such as those detectable in *ob/ob* or antagonist administered mice, are associated with an increase in the amount of lipid storage (Coleman, 1978; Verploegen et al., 1997). Because of above listed evidence, leptin is considered an anti-obesity hormone, preventing weight gain in humans and animals (Farooqi and O'Rahilly, 2009; Flier and Maratos-Flier, 2010; Friedman, 1998).

As demonstrated by Quian et al. (1997), leptin also induces also apoptosis of adipocytes in rats and enhances the activation of the Hormone Sensible Lipase (HSL), the rate limiting enzyme of the cholesterol ester degradation pathway in macrophages

(O'Rourke et al., 2001). This latter effect should markedly inhibit the cholesterol ester storage in these cells, preventing its accumulation on the walls of blood vessels and foam cell formation, which is considered as the initiation of atherosclerosis.

A growing body of evidence suggests that energy balance and weight are not only under leptin control, but, more properly, depend on its interaction with other hormones, such as insulin and glucocorticoids (Schwartz and Seeley, 1997). In a previous study conducted by Coleman (1978) leptin was shown to interfere with glucose metabolism: *ob/ob* mice are, in fact, diabetic and the severity of the disease depends on the starting genotype. Notably, hyperglycemia and leptin-resistance are recovered by exogenous administration of the lacking hormone (Cummings et al., 2011).

The improvement in glucose homeostasis, which is observed after leptin administration in mice, is mostly due to the central effects of this hormone. At peripheral level, conversely, leptin shows insulin-like effects, facilitating both transport and biosynthetis of glycogen, most likely through the activation of JAK-2 and insulin receptor substrate (IRS)-2 (Berti et al., 1997; Kellerer et al., 1997).

Furthermore, an increased sympathetic activity has been reported in brown adipose tissue (BAT) from leptin administered *ob/ob* mice (Collins et al., 1996); this effect has been associated to an increase of the thermogenesis, since leptin administration enhances oxygen consumption and leads to an induction of uncoupling protein (UCP)-1, -2 and -3 expression (Scarpace et al., 1998). These proteins uncouple mitochondria, producing high rates of substrate oxidation and an increase in heat production without the phosphorylation of adenosine 5'-diphosphate (ADP).

Moreover, this hormone has an angiogenic activity due to the activation of Ob-Rb in endothelial cells (Sierra-Honigmann et al., 1998): leptin, secreted from adipocytes into

the bloodstream, acts locally at endothelial cells level (paracrine effect) by inducing angiogenesis. This effect could be important for the dissipation of heat in the sites where thermogenesis is active.

5.2.6 Leptin-resistance

Leptin modulates 5' adenosine monophosphate-activated protein kinase (AMPK) activity and orexigenic/anorexigenic neuropeptides in discrete hypothalamic regions (Minokoshi et al., 2002; Mountjoy et al., 2007), leading to a reduction of appetite, and to an overall negative energy balance. Indeed, obesity is often accompanied by leptin resistance. This latter phenomena is defined as decreased sensitivity to the anorexigenic or weight loss effects of leptin accompanied by increased serum level of the hormone.

Generally, obese individuals are leptin-resistant since they show high levels of body fat despite the presence of hyperleptinemia. It has been demonstrated that the administration of the recombinant leptin reduces body fat in obesity murine models. This effect seems to be minimal in obese humans. The injection of recombinant leptin reduces appetite, but has no effect on energy expenditure or basal metabolism. These data suggest that leptin-resistance occurs primarily in metabolically active tissues, such as skeletal muscle (Westerterp-Plantenga et al., 2001). The development of leptin-resistance is characterized by the suppression of the rate of AMPK activation (Steinberg et al., 2004). SOCS3 and protein-tyrosine phosphatase (PTP) 1B are two well-known mediators of leptin resistance (Bjørbaek et al., 1998; Zabolotny et al., 2002). As reported by Bjørbaek et al. (2001), as a consequence of the interaction between leptin and its receptor Ob-Rb, the phosphorylation of two tyrosine residues, through the activation of JAK2, occurs. The

activation of STAT3, through the phosphorylation by JAK2, leads to the activation of the transcription of SOCS3 encoding gene.

SOCS3 then phosphorylates Ob-Rb resulting in the inhibition of this recptor signalling transduction. To date, SOCS3 is a member of the only known inducible inhibitors of cytokine signalling protein family which consists of the (SOCS) proteins, of which there are eight family members: SOCS1–SOCS7 and the cytokine-inducible SH2-domain-containing protein (CIS). All these proteins bind with their SH2 domains to phosphotyrosine residues in cytokine receptors (in the case of SOCS2, SOCS3, and CIS) (Yoshimura, 2004) or JAKs and thus suppress cytokine signalling either by binding to or inhibiting the activity of JAKs, by competing with STATs for the phosphorylated binding sites on receptors, or by targeting bound signalling proteins for proteasomal degradation (Wormald and Hilton, 2004).

At hypothalamic level, this protein inhibits the signal transduction mediated by STAT3 trough the binding to the Tyr_{985} residue of Ob-Rb (Bjørbaek et al., 1999; 2001). Interestingly, neural cell-specific SOCS3 conditional knockout mice, generated using the Cre-loxP system, showed a restoration of leptin sensitivity (Mori et al., 2004).

SOCS3 is not only involved in the impairment of leptin sensitivity at central level, but it also plays a crucial role in the development of leptin resistance at peripheral level, as shown by its expression increase in skeletal muscle from rats on high fat diet (Steinberg et al., 2004). Moreover, SOCS3 inhibits AMPK activation in peripheral metabolically active tissues, such as liver, WAT and skeletal muscle, contributing to abnormalities of fatty acid metabolism. Besides SOCS3, PTP1B is a negative regulator of both leptin and insulin signalling (Fig. 15).

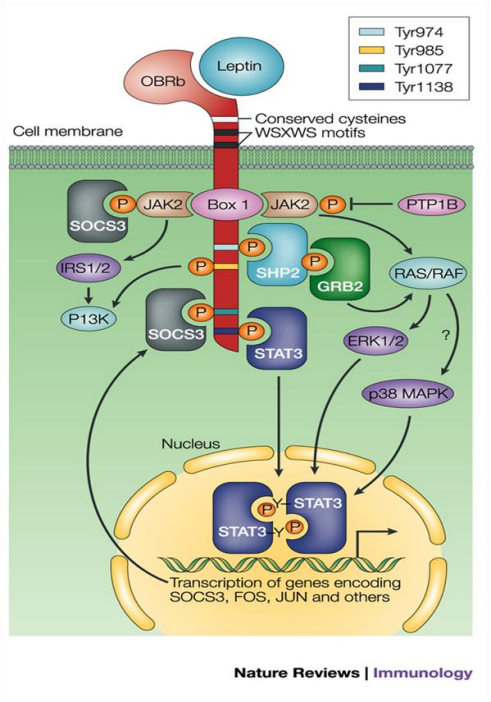


Fig. 15 Mechanism of leptin signalling inhibition by suppressor of cytokine signalling 3 (SOCS3) and phosphotyrosine phosphatase 1B (PTP1B). After binding leptin, OBRb-associated JAK2 becomes activated by auto- or cross-phosphorylation and tyrosine phosphorylates the cytoplasmic domain of the receptor. Four of the phosphorylated tyrosine residues function as docking sites for cytoplasmic adaptors such as STAT factors, particularly STAT3 (in some cases, also STAT1 and STAT5). The membrane distal tyrosine (position 1138) functions as a docking site for STAT3, which is a substrate of JAK2. After subsequent dimerization, STAT3 translocates to the nucleus and induces the expression of SOCS3 and other genes. SOCS3 takes part in a feedback loop that inhibits leptin signalling by binding to phosphorylated tyrosines. COnveresely, PTP1B, which is localized on the surface of the endoplasmic reticulum, is involved in negative regulation of OBRb signalling through the dephosphorylation of JAK2 after internalization of the OBRb complex.

This phosphatase dephosphorylates JAK2, the first kinase involved in signal transduction via ObRb, reducing the hormone sensitivity. Mice lacking the PTP1B are more sensitive to leptin and resistant to obesity (Zabolotony et al., 2002; Klaman et al., 2000; Bence et al., 2006). Due to its prominent role in regulating metabolism, PTP1B is a promising therapeutic target for the treatment of obesity, as well as type II diabetes mellitus. PTP1B is encoded by the PTPN1 gene on human chromosome 20q13, a region that shows linkage with insulin resistance, type 2 diabetes, and obesity in human populations (Tsou and Bence, 2012).

5.2.7 AMP-Activated Protein Kinase: catabolic and anti-inflammatory effects

AMPK is ubiquitously expressed and it has now become clear that it plays a wide role in cellular regulation and in the control of fatty acid oxidation in liver (Muoio et al., 1999) and muscles (Vavvas et al., 1997), in the activation of glucose uptake by muscles (Hayashi et al., 1998), and in the inhibition of glucose-activated gene expression in the liver (Leclerc et al., 1998). AMPK is a fuel-sensing enzyme with a peculiar catabolic activity due to the promotion of ATP production, thereby restoring the AMP/ATP ratio.

AMPK phosphorylates and inhibits key enzymes involved in biosynthetic pathways (Fig. 16), such as ACC (fatty acid synthesis) and 3-hydroxy-3-methylglutarylCoA reductase (isoprenoid and cholesterol biosynthesis) (Lopez et al., 2007a). AMPK enhances the fatty acid oxidation, reducing thus malonyl-CoA levels through the ACC2 inhibition (Lopez et al., 2007a).

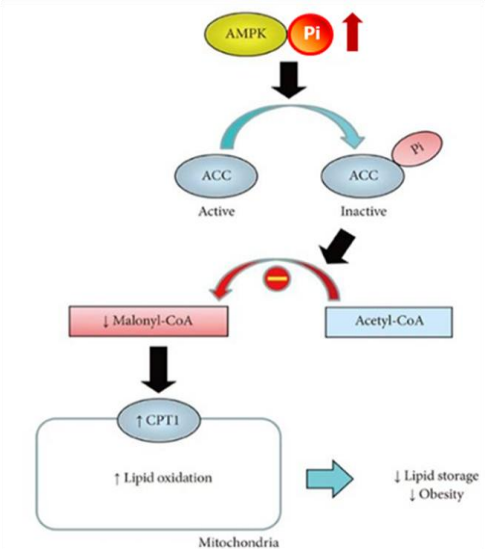


Fig. 16 Regulation of fatty acid metabolism in peripheral tissues, such as white adipose tissue (WAT). Acetyl CoA carboxylase (ACC) is inactivated upon phosphorylation by pAMPK. Active ACC catalyzes the carboxylation of acetyl CoA to malonyl CoA, which inhibits the rate-limiting enzyme in mitochondrial fatty acid uptake, carnitine palmitoyltransferase (CTP) 1.

This latter effect drives the disihnibition of carnitine palmitoyltransferase (CPT) 1 and, as a consequence, the increase in the rate of fatty acid oxidation (Lopez et al., 2007a). Despite its involvement in the control of energy homeostasis, the role of AMPK in adipose tissue remains controversial. Initial studies have described an anti-lipolytic role for AMPK, whereas more recent studies have suggested the converse.

In particular Daval et al. (2005) have presented data suggesting that AMPK acts on hormone-sensitive lipase by blocking its translocation to the lipid droplet and have concluded that, in mature adipocytes, AMPK activation has a clear anti-lipolytic effect.

Previous results may suggest that an acute bout of low-intensity prolonged swimming exercise directly enhances the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) mRNA expression in the activated muscle during exercise, possibly through an AMPK-related mechanism (Terada et al., 2002). Moreover, Zong et al. (2002) have demonstrated that AMPK is also involved in initiating mitochondrial biogenesis by sensing the energy status of the muscle cell.

AMPK has been proposed to be an important intracellular energy sensor because this enzyme controls both lipid and glucose metabolism. In fact, Holmes et al. (1999) have provided several observations which, taken together, are consistent with the hypothesis that the increase in skeletal muscle glucose transporter (GLUT)-4 and hexokinase II induced by training are mediated by AMPK activation.

To date, this enzyme has been recognized to play a key role in coordinating the CNS and the peripheral tissues to achieve energy balance (Perrin et al., 2004); but most remarkably recent data suggest a strong correlation between the AMPK activation and the inhibition of the proinflammatory cytokine synthesis, leading to the occurrence of macrophage polarization towards the lean phenotype (Lihn et al., 2008).

In response of several stimuli, mononuclear phagocytes express specialized and polarized functional properties and, as a consequence, we can currently refer to polarized macrophages as M1 and M2 cells, mirroring the Th1/Th2 nomenclature (Mantovani et al., 2005). Classically activated M1 macrophages have long been known to be induced by interferon (IFN)-γ and to produce proinflammatory cytokines, such as IL-1β, IL-6 and TNFα. On the opposite site, alternatively activated M2 macrophages generally have high levels of scavenger, mannose, and galactose-type receptors, and arginine metabolism is shifted to production of ornithine and polyamines via arginase (Lumeng et al., 2007). Besides the cytokines, also the diet-induced obesity leads to a shift in the activation state of adipose tissue macrophages (ATMs) from a M2-polarized state in lean animals, that may protect adipocytes from inflammation, to a M1 proinflammatory state that contributes to insulin resistance (Lumeng et al., 2007).

5.3 PCB exposure and osteoarthritis

PCBs cause a wide range of toxic effects, such as immune suppression, endocrine and metabolic disruption.

Endocrine disruptors, such as PCBs, markedly influence the immune system, increasing the risk of autoimmune diseases, such as rheumatoid arthritis (RA). In addition, beyond the negative effects of increased weight bearing caused by obesity, metabolic change due to adipose tissue has recently been proposed as one underlying mechanism of Osteoarthritis (OA; Conde et al., 2011). OA pathogenesis has been recently associated with adipokines (Scotece et al., 2013). In particular, Otero et al. (2005) have demonstrated that leptin could directly participate to the damage of joints, causing the OA onset and progression. Several studies have shown that PCBs, mostly NDL-congeners may

upregulate leptin levels, probably leading to an obesogenic effect (Wahlang et al., 2013; Taxvig et al., 2012). Thus, taking into account that, OA pathogenesis is associated with adipokines and that NDL-PCBs alter adipokines expression, it is plausible to speculate that these pollutants could be involved in the pathogenesis and/or progression of OA.

However, only few studies have analyzed the correlation between exposure to PCBs and pathogenesis of OA. Most of these studies have found a strong positive correlation between serum levels of PCBs as a consequence of environmental poisoning, and the incidence of swelling of the joints and arthralgia (Kanagawa et al., 2008), as well as of OA (Guo et al., 1999), mainly in women (Lee et al., 2007).

Destruction of the cartilage matrix by a pathological imbalance of normal chondrocyte function is a key element in OA progression. Chondrocytes are the only cell type in articular cartilage and play an essential role for the integrity of the cartilage. Thus, chondrocyte cell death leads, a logic consequence, to cartilage damage, which is the most prominent feature of OA. Chondrocyte apoptosis is more frequently observed between advanced OA cases than in normal subjects. Since apoptotic cell death is a critical event in the pathogenesis of joint diseases, identification of apoptosis inducers is considered a key element in understanding the causes of OA.

Several in vitro studies have shown that exposure to PCB commercial mixtures (i.e. Aroclor) leads to a decrease in cell viability and acceleration of apoptosis of neurons (Mariussen et al., 2002), nephrons (Pérez-Reyes et al., 2001), and splenocytes (Yoo et al., 1997). Also the individual congeners have been long known to be able to induce apoptosis in different cell lines. In particular, PCBs 153, 118 and 74 induce apoptosis in the immortalized hypothalamic GT1-7 cell line, which synthesizes the neuroendocrine peptide gonadotropin-releasing hormone (GnRH) (Dickerson et al., 2009).

Sànchez-Alonso et al. (2003) have highlighted the NDL-PCB 153, as well as the DL-PCB 77, apoptosis-mediated neurotoxic potential in neuronal cell cultures. Consistently, the study conducted by Howard et al. (2003) indicates that NDL-PCBs induce apoptosis in hippocampal neurons subsequent to ryanodine receptor (RyR) activation and ROS increase and suggests that altered profiles of apoptosis in specific anatomic compartments may be an important mechanism underlying the developmental neurotoxicity of PCBs. Moreover, PCBs 153 and 126 have also been shown to induce apoptosis in AtT20 pituitary cells (Jhoansson et al., 2006).

Regarding immunotoxicity, our previous study has demonstrated that the NDL-PCBs 101, 153 and 180, alone or in combination, induce apoptosis of J774.A1 murine macrophages through the intrinsic pathway of cell death process (Ferrante et al., 2011). It is noteworthy, finally, that PCB 126, the most potent DL-congener, has been shown to induce apoptosis of chondrocytes via a ROS-dependent pathway (Lee et al., 2012). Conversely no further study has evaluated the proapoptotic effects of NDL-PCBs on chondrocytes, and established their mechanism of action.

5.3.1 Apoptosis

It has been almost three decades since the term apoptosis was coined by Kerr (1972) and is a synonymous of the term "programmed cell death " introduced by Lockshin in 1964. It describes a unique form of cell death that orderly involves gene-dependent cell disintegration. Apoptosis is a distinctive form of cell death exhibiting specific morphological and biochemical characteristics, including cell membrane blebbing, chromatin condensation, genomic DNA fragmentation, and exposure of specific

phagocytosis signaling molecules on the cell surface. Regulation of this process has been associated to the onset of neoplastic and/or autoimmune diseases.

Cells undergoing apoptosis differ from those dying through necrosis. It is accepted that there are two major pathways of apoptotic cell death induction: extrinsic signaling through death receptors, that leads to the formation of the death-inducing signalling complex (DISC), and intrinsic signalling mainly through mitochondria that leads to the formation of the apoptosome.

5.3.2 Apoptosis and Necrosis

Apoptosis, dissimilar to necrosis, is not a silent process and is carried out from the cells, transcribing specific genes, synthesizing new enzymes and consuming energy. Finally, apoptotic cells might influence neighbouring cells trough the synthesis of specific mediators or cell-cell interaction (Arnoult et al., 2002). Necrosis, on the other hand, occurs as a result of serious and irreversible pathological damage such as hypoxia, hyperthermia, viral infections, exposure to various toxic agents or complement activation (Israels and Israels, 1999). These events lead to the loss of integrity of the cellular membranes, including that of the nucleus, resulting in the release of their contents (ATP, proteases and lysozymes), in the entire cell break and in the DNA degradation.

Necrotic cells are usually recognized by the immune system as danger signal and, thus, resulting in inflammation; in contrast, apoptotic death is quiet and orderly. During apoptosis, on the opposite, the cells are condensed, because the cytosolic volume decreases and the loss of membrane specializations occurs in the same time of the detach from neighboring cells. Apoptosis, at least in the early stages, is not able to trigger an inflammatory response, because, dissimilar to necrosis, does not determine the release of

the cytosolic content in surrounding tissues. In fact, initially the integrity of the cytosolic organelles is preserved, while at nuclear level, the disintegration of the nucleus, both condensation and fragmentation of chromatin in portions of 180-200 base pairs or multiples occurs (Fig. 17).

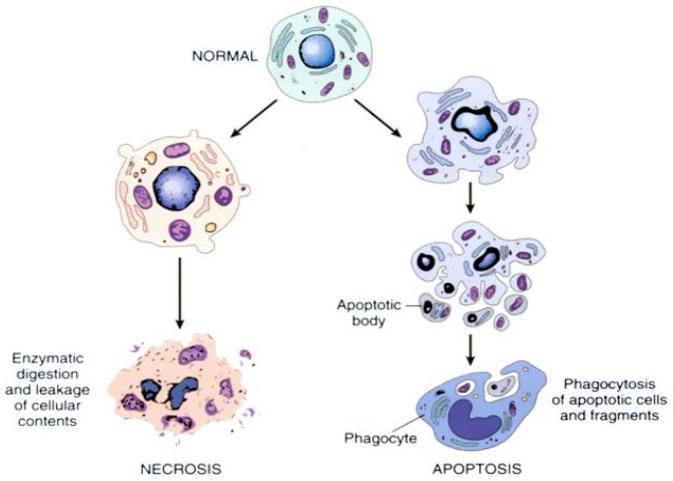


Fig. 17 Structural changes of cells undergoing necrosis or apoptosis.

Stereotypical morphological changes occur in almost all cells undergoing apoptotic death. The chromatin, notably, is degraded in compact granules, moving from the periphery of the core toward the plasmamembrane. Here, the above mentioned granules are surrounded by cytosolic membrane vesicles, making the apoptotic cells similar to bubbles. Besides the cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation, all signs of apoptosis include, finally, the formation of separated vesicles called "apoptotic bodies" (Fig. 18).

Apoptosis can be induced by various stimuli, both intra- and extra-cellular, which activate different pathways. Among these, the extrinsic pathway needs the expression of plasmatic receptors and involves the activation of caspase-8, but the intrinsic pathway affects the mitochondria functions and involves the activation of caspase-9 (Hengartner, 2000).

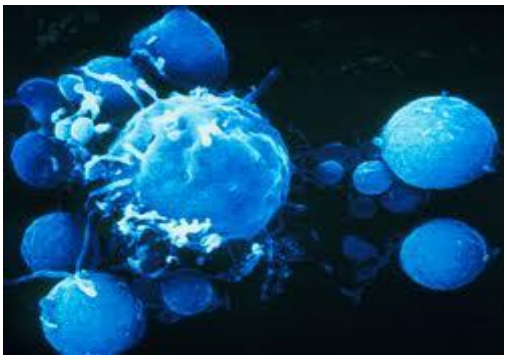


Fig. 18 Morphology of a cell undergoing apoptosis surrounded by "blebs."

The extrinsic pathway of apoptosis signalling is initiated when death receptors (DRs) at the cell surface encounter specific cognate “death ligands,” inducing a conformational change that is transmitted within the cell membrane. Three major specific cell DR/ligand pairs have been described, all members of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF): (1) Fas and Fas ligand (FasL) (Fas is also called Apo-1, CD95 or TNFRSF6 (Itoh et al., 1991); FasL is also called CD178, CD95L or TNFSF6 (Suda et al., 1993); (2) DR4 and DR5 and TNF-related apoptosis inducing ligand (TRAIL, also called Apo2L or TNFSF10) (Pitti et al., 1996); and (3) TNFα and the TNF-R1. The TNF, binding, for example, to the TNFR-1, can determine the activation of caspase-8 and triggers the extrinsic pathway. This is a complex process that requires the trimerization of the receptor, recruiting the death domain (DD) and two adaptor proteins (TRADD and FADD/MORT-1). The FADD/MORT-1 protein, in turn, binds to procaspase-8, resulting in its activation (Hengartner, 2000).

5.3.3 Intrinsic apoptosis signalling pathway

The mitochondrial pathway of apoptosis is activated in response to both internal, such as DNA damage, and extracellular, such as the lack of growth factors, signals. These stimuli converge on mitochondria and lead to the release of cytochrome c, the second

mitochondria-derived activator of caspases (SMAC, also termed DIABLO), the apoptosis-inducing factor (AIF), which is related to chromatin condensation and endonuclease G upregulation.

The mitochondrial outer membrane permeabilization (MOMP), considered the point-of-no-return in apoptosis induction, is a key event in the intrinsic pathway. Release of certain proteins from the mitochondrial intermembrane space due to MOMP triggers a cascade of caspase activation that results in irreversible events culminating in apoptosis. MOMP is normally prevented by anti-apoptotic members of the Bcl-2 family, which is composed of both anti-apoptotic and pro-apoptotic proteins.

Among the pro-apoptotic proteins, Bax appears to be requisite for MOMP. Once activated, Bax permeabilizes the outer membrane of mitochondria, resulting in release of pro-apoptotic factors such as cytochrome c. In particular, once MOMP occurs, proteins in the intermembrane space are released to the cytosol. One such protein, cytochrome *c*, binds to cytosolic, monomeric apoptotic protease activating factor-1 (APAF-1). This interaction promotes the APAF-1 oligomerization to initiate the apoptosome formation. The apoptosome then binds to the proform of caspase 9, the initiator caspase of the intrinsic pathway, via the caspase recruitment domains in both APAF-1 and pro-caspase 9. Caspase activity can be modulated by caspase-binding proteins of the inhibitor of apoptosis proteins (IAPs) family (Breckenridge and Xue, 2004). Cytochrome c is released into the cytosol through different mechanisms. Among these, one mechanism consists in cytochrome c dissociation from cardiolipin, through which is normally located at inner mitochondrial membrane level. This release can be stimulated by the production of ROS as well as by the binding of Ca^{2+} to cardiolipin (Orrenius, 2003).

Previously studies have demonstrated the involvement of two pro-apoptotic proteins, Bax and Bak, in the regulation of the intracellular amount of Ca^{2+} (Petronilli et al., 2001). Bax and Bak translocate from the cytosol to the endoplasmic reticulum (where they cause a decrease in Ca^{2+} levels) and to the mitochondria (where they increase the Ca^{2+} internalization), causing changes in MOMP (Petronilli et al., 2001). Both intrinsic and extrinsic pathway lead to the activation of effector enzymes called caspases (cysteine aspartate proteases), whose main role is the amplification of apoptotic signals (Fig. 19).

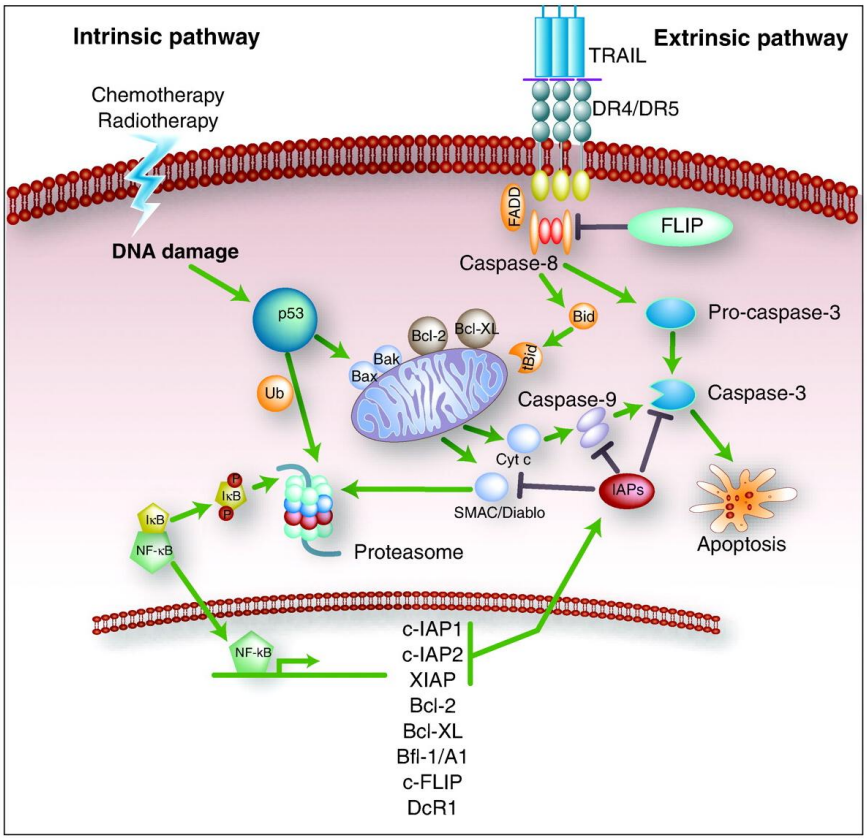


Fig. 19 Schematic representation of the intrinsic and extrinsic apoptotic pathway. Apoptosis induction via the death receptor can result in activation of the extrinsic and intrinsic pathways.

Chapter 3

6. MATERIALS AND METHODS

6.1 Bioaccumulation in two bivalve species

6.1.1 Study area and sampling collection

Farmed specimens of Mytilus galloprovincialis and wild specimens of Ensis siliqua were collected during the period May-July 2008 whithin that part of the Tyrrhenian Sea which is adjacent to the coastal area of Castelvolturno (in the province of Caserta) (Fig. 19).

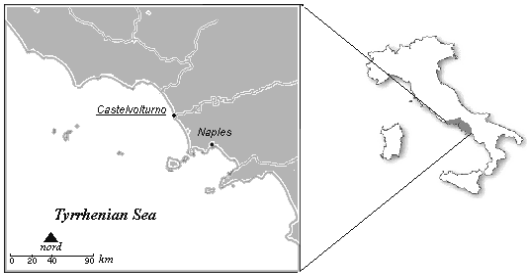


Fig. 19 Location of the costal area of Castelvolturno (Caserta).

This area receives waters of the Volturno river, the most important river in south of Italy in terms of length and drainage basin, that may contribute to the marine pollution (Parrella et al. 2003). The Mediterranean mussels were collected at 5 m of depth from a wooden pike in a rectangular area located about 5 Km far from the coast (between N41°/03'/43 and N41°/02'/60 and between E13°/51'/30 and E13°/52'/00, respectively, for latitude and longitude). The razor clams were collected by hand in shallow sea waters few meters far from broad sandy beaches. The specimens, all of commercial and homogeneous size, were immediately refrigerated, and transported to the laboratory.

6.1.2 Analytical sample preparation

After washing the surface crust, lengths and weights of the molluscs were measured and recorded. Just after having collected a given group of specimens, the bivalves were cracked through excision of the adductor muscle and the soft tissues were removed and pooled to form our sample unit of about 100 g of weight. The samples of mussels and clams consisted of 40 and 15 units, respectively. Each unit was homogenized and stored at -20 °C until chemical analysis.

6.1.3 Chemical analysis

Concentration levels of 5 OCPs – HCB, Dieldrin, p,p'-DDT, 1,1'-dichloro-2,2'-bis(4-chlorophenyl) ethylene (p,p'-DDE) and 1,1'-dichloro-2,2'-bis-(4-chlorophenyl)-ethane (p,p'-DDD) – and 20 PCBs – IUPAC nos. 28, 52, 66, 74, 99, 101, 105, 118, 128, 138, 146, 153, 170, 177, 180, 183, 187, 196, 194, and 201 – were determined for each sample unit.

Among the above mentioned PCBs there are some congeners (IUPAC nos. 28, 52, 101, 118, 138, 153, 180) that have been proposed by the International Council for the Exploration of the Sea (ICES) as indicators of PCBs contamination (ICES, 1992). The other investigated PCBs were chosen because they were known to be markedly bioaccumulated in fish-eating birds from the Mediterranean Sea and the Atlantic Ocean (Walker, 2001).

The extraction and separation of the analytes from the lipid fraction, and the purification of the extracts were carried out adapting the method described by Di Muccio et al. (2002) as shown in detail in a previous paper (Naso et al., 2005) . For each sample unit, an aliquot of about 3 g was manually cold-extracted for 2 min with 25 mL of petroleum

ether/acetone (1:1, v/v), and centrifuged at 1600 rpm for 12 min. The extract was passed through a glass tube packed with anhydrous sodium sulphate and then evaporated to dryness by rotavapor. The extracted Lipid-content was determined gravimetrically. The lipid dried extracts were re-suspended in n-hexane, transferred on Extrelut-3/Extrelut-1 cartridges (Merck Kga A Darmstad, Germany) with the addition of 0.36 g of C-18 Isolute (40–60 mesh Merck Kga A Darmstad, Germany) and eluted with acetonitrile. The extracts, concentrated at 1 mL under vacuum at 40 °C, were cleaned up on a glass column containing 2.5 g of activated Florisil (60/100 mesh – Supelco Bellefonte, PA USA). The column was eluted with 30 mL of n-hexane, 25 mL of n-hexane–toluene (80:20, v/v) and 30 mL of n-hexane–toluene–ethyl acetate (180:19:1, v/v/v). The first fraction contained PCBs, HCB and p,p'- DDE, the second one contained p,p'-DDD and p,p'-DDT while the Dieldrin was present in the last fraction. The three fractions were concentrated to a small volume and PCB 209 not found in the environmental samples was added as an internal standard for the GC–ECD analysis.

Gas chromatographic analysis of the PCBs and OCPs were carried out by a Carlo Erba HRGC 5160 Mega Series equipped with a ^{63}Ni electron capture detector. The cold on-column mode was used for the injection. Two fused silica capillary columns of different polarities coated with a CP-SIL 5CB (25 m×0.32 mm id, 0.25 μm film thickness) (Varian Inc., UK) and Rtx-1701 (30 m×0.32 mm id, 0.25 μm film thickness) (Restek, UK) were used to separate and quantify the residues. The ECD was kept at 310 °C. Hydrogen and nitrogen were used as carrier gas and make-up gas, respectively. Multi-level calibration curves were created for the quantification and good linearity (r^2>0.998) was achieved for tested intervals that included the whole concentration range found in samples. Organochlorines were identified by comparing the retention times on the two columns with

those of the corresponding standard compounds and then quantified by comparing the individually resolved peak areas with those of the corresponding standards.

The total value of the seven indicator-PCBs were added to the other PCBs to obtain the sum of PCBs (indicated as ∑PCBs). ∑DDTs were calculated as the sum of p,p'-DDT, p,p'-DDE and p,p'-DDD. Data, not corrected for recovery, were expressed as ng g^{-1} lipid weight (LW) and ng g^{-1} soft tissues wet weight (WW). For all PCBs and OCPs the detection limits ranged from 0.08 to 0.60 ng g^{-1} LW. Results were reported as not detectable (nd) when the concentrations were lower than the detection limits.

Pure reference standard solutions were used for instrument calibration, recovery determination, and quantification (Dr. Ehrenstorfer laboratory, Augsburg, Germany). All the solvents were pesticide residue analysis grade, purchased from Pestiscan (Labscan, Dublin, Ireland).

Mussel edible tissue blanks were extracted and analysed to check for cross-contamination. In addition, aliquots of the blanks, spiked with standard mixtures at three concentration levels, were extracted and analysed in triplicate to evaluate the recovery of each contaminant. The method limits of quantification for individual PCBs and OCPs were between 0.1-0.3 and 0.1-0.2 ng g^{-1} LW, respectively. The recovery rate of the method for OCPs was in the range 80%-108% and for PCB congeners it was between 91%-110% depending on the congener. Certified reference materials (mussel tissues homogenate) supplied by the National Institute of Standards and Technology (SRM 2977 and SRM 1974b) were used for quality control.

6.1.4 Statistical analysis

Statistical analysis was carried out on the lipid-normalized organochlorine concentrations and on concentration expressed on WW. Data were preliminary analysed through summary statistics such as median, mean and standard deviation. Differences in concentrations of OCs among the two species were evaluated by sample means difference test (*t*-Student statistics). When the P-value was less than 0.05, the difference was considered statistically significant. Further, the one-way analysis of variance (ANOVA) and the Tukey–Kramer Multiple Comparisons Test provided some comparisons with results relative to previous studies. All analyses and calculations were performed by GraphPad Software (GraphPad InStat).

6.2 Experiments in J774A.1 murine macrophages

6.2.1 Reagents

2,2′,4,5,5′-Pentachlorobiphenyl (PCB 101), 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB 153) and 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB 180) (99% purity) were obtained from Sigma Aldrich (St. Louis, MO). All PCBs were dissolved in dimethyl sulphoxide (DMSO) in a 10 mM stock solution. Dulbecco's modified Eagle's medium (DMEM) without Red Phenol, Fetal bovine serum (FBS), tissue culture media and supplements were purchased from Lonza (Walkerville, MD, USA). *Escherichia coli* lipopolysaccharide (LPS, serotype 0111:B4) was purchased from Fluka (Milan, Italy). The antibody against cyclooxygenase (COX)-2 was obtained from Cayman Chemical (Ann Arbor, MI), while the antibody against inducible nitric oxide synthase (iNOS) was acquired by BD Biosciences Transduction Laboratories (Lexington, KY) and the antibodies against IkB-α

and the nuclear factor (NF)-kB by Santa Cruz Biotechnology (Santa Cruz, CA). Unless stated otherwise, all reagents and compounds were obtained from Sigma Chemicals Company (Sigma, Milan, Italy).

6.2.2 Cell lines and culture conditions

The J774A.1 cell line (BALB/c murine macrophages) was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, U.K.) and cultured as previously described (Ferrante et al., 2011). In all experiments the cells were mechanically scraped and plated. After 4 h to allow adhesion, cells were starved in 5% FBS Red Phenol free DMEM for 2 h and subsequently treated with PCB 101, 153 or 180 alone (100 nM) or in combination (100 nM) for different times depending on the assay in presence of LPS (10 ng/mL). The final concentration of DMSO in all samples was 0.1% (v/v). This DMSO percentage allows the optimal solubilization of PCBs in aqueous solutions. The nanomolar concentrations of PCBs were chosen because they did not significantly modify cell viability.

6.2.3 Determination of cell viability

The viability of macrophages was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to detect functional mitochondria in living cells (Mosmann, 1983). Functional mitochondria can transform MTT to formazan salts, which can be measured with spectrophotometer. The proportion of viable cells in treated samples was compared with control samples. Cells (3×10^4/well) were plated on 96-well microtiter plates to a final volume of 150 μL. After 24 h of incubation with PCBs at increasing concentrations (100 nM-10 μM) alone or in presence of LPS (10 ng/mL) at 37° C, 25μL of

MTT (5 mg/mL) were added to each well and the cells were incubated for an additional 3 h. Thereafter, cells were lysed with 100µL of a solution containing 50% (v/v) N,N-dimethylformamide, 20% (w/v) sodium dodecyl sulphate (pH 4.5) to allow solubilization of dark blue crystals. Then, after 20 h incubation at 37 ◦C, the optical density (OD_{620}) of the samples treated with the different serial dilutions of PCBs alone or in combination with LPS were compared with the OD of control wells to assess the cell viability, which was calculated as: % dead cells = 100− (OD treated/OD control)×100.

6.2.4 Real-time semi-quantitative PCR analysis

After 4 h of incubation with PCBs, alone or mixed (100 nM), in presence of LPS (10 ng/mL), total RNA was extracted by a modified method of Chomczynski and Sacchi (1987), using Trizol Reagent (Invitrogen Biotechnologies) in accordance with the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 µg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories) as previously described (Mattace Raso et al., 2014). The primer sequences are reported in Table 5.

Table 5 Real Time PCR Primer Sequences

Target gene	Forward primer (5'⟶ 3')	Reverse primer (3'⟶5')	Accession Number
IL-6	5'-ACAAGTGGGAGGCTTAATTACACAT-3'	3'-TTGCCATTGCACAACTCTTTTC-5'	NM_031168.1
TNFα	5'-CATCTTCTCAAAACTCGAGTGACAA-3'	3'-TGGGAGTAGATAAGGTACAGCCC-5'	NM_013693.3
MCP-1	5'-CCCACTCACCTGCTGCTACT-3'	3'-TCTGGACCCATTCCTTCTTG-5'	NM_011333.3
GAPDH	5'-AACTTTGGCATTGTGGAAGG-3'	3'-GGATGCAGGGATGATGTTCT-5'	NM_008084.2

The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample

contained 1-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer (Eurofins MWG Operon, Huntsville, AL) in a final volume of 25 µl. The relative expression of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

6.2.5 Western blot analysis

COX-2 and iNOS expression: after 24 h of incubation with PCBs, cells were washed twice with ice cold PBS, harvested, and resuspended in 20 mM Tris–HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), leupeptin and trypsin inhibitor (10 µg/mL). After 1 h, cell lysates were obtained by centrifugation at 20,000×g for 15 min at 4 ◦C.

IkB-α and NF-kB expression: To evaluate the temporal effect of LPS on IkB-α degradation and nuclear p65 NF-kB expression and the effects of PCBs, alone or in combination, on LPS-induced IkB-α degradation and nuclear p65 NF-kB content, nuclear and cytosolic protein lysates were obtained. J774A.1 cells were treated with LPS (10 ng/mL) for 0–5–15–30 min or, in another set of experiments with PCBs, alone or mixed, in presence of LPS for 30 min. At the determined times, cells were suspended in extraction buffer (0.32 M sucrose, 10 mM Tris–HCl pH 7.4, 1 mM ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid [EGTA], 2 mM ethylenediaminetetraacetic acid [EDTA], 5 mM NaN_3, 10 mM 2-mercaptoethanol, 50 mM NaF, 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, 1 mM Na_3VO_4), scraped off, harvested on ice for 15 min and then centrifuged at 1000 g for 10 min, 4 ◦C. IkB-α degradation was evaluated in the cytosolic

supernatant fraction. The pellets were resuspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM Na_3VO_4 and then centrifuged 30 min at 15,000 g at 4 ◦C to yield the nuclear fraction for the p65 NF-kB level determination.

Protein concentrations were estimated by Bio-Rad protein assay using bovine serum albumin as standard. Equal amount of protein (cell lysates) were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The filter was, then, blocked with 1× PBS, 5% nonfat dried milk and incubated with specific antibodies in 1× PBS, 5% nonfat dried milk, 0.1% Tween-20 for 2 h at room temperature. We used the specific mAbs against COX-2 (1:500), iNOS (1:1000), IkB-α (1:2000) and p65 NF-kB (1:500) in PBS, 5% non fat dried milk, 0.1% Tween 20 at 4 ◦C, overnight. After incubation with the primary antibody and washing in 1×PBS, 5% nonfat dried milk, 0.1% Tween, the secondary antibody (IgG-horseradish peroxidase conjugate; 1:2000 dilution) was incubated for 1 h at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions, and the immune complex visualized by Imag Quant. The protein bands were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Milan, Italy). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibodies against the GAPDH or β–actin protein (Sigma-Aldrich, St. Louis, MO).

6.2.6 Measurement of nitrite in supernatants

After 2 hr of starvation in 5% FBS Red Phenol free DMEM, cells (10^6/P60 dish), were treated with NDL-PCBs, alone or differently associated (100 nM), in presence of LPS (10 ng/mL). The concentration of compounds was chosen on the basis of the experiments performed on cell viability. Pollutants were first dissolved in absolute DMSO and then diluted with DMEM. The final DMSO concentration in all well was 0.1%. After 24 hr pollutant exposure, NO^{2-} was measured in the supernatant. Griess reaction was used to measure nitrite, one of the stable end products of nitric oxide. 100 µL of cell culture supernatant was added to 100 µL Griess reagent [0.1% (w/v) naphtylethylenediamine-HCl and 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid (vol.1:1)]. The optical density at 550 nm was measured using a microplate reader Titertek. Nitrite concentrations were determined by linear regression from a sodium nitrite standard curve freshly prepared in culture medium and expressed as micromolar unit. Triplicate measurements were averaged from three independent experiments.

6.2.7 Statistics

Data are reported as mean ± standard error mean (S.E.M.) values of independent experiments, which were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test or, when appropriate, with Dunnet's test. Statistical significance was set at $P<0.05$.

6.3 Experiments in differentiated 3T3L-1 cell line

6.3.1 Chemicals

Insulin (INS), dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), Oil Red O (ORO), isopropanol and DMSO were purchased from Sigma Chemicals Company (Sigma, Milan, Italy).

6.3.2 Adipogenic differentiation and cell treatment

3T3-L1 mouse fibroblast cells, purchased from European Collection of Animal Cell Cultures (Salisbury, Wiltshire, U.K.), were maintained in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin and cultured in 75-cm^2 cell culture flasks. Cultured cells were allowed to grow to 100% confluence at 37°C in a humidified 5% CO_2 atmosphere with media changes every 2-3 days. The standard procedure of adipogenic differentiation is shown in Fig. 20. 3T3-L1 cells were cultured to confluence. At the confluence (ID 0), the cells were incubated in adipogenesis-inducing medium (MD I) (DMEM containing 10 μM DEX, 0.5 mM IBMX, 10 μg/mL Ins and 10% FBS) for 2 days (ID 2), then in adipogenesis maintaining medium (MD II) (DMEM containing 10 μg/mL INS and 10% FBS) for 2 days (ID 4), followed by DMEM with 10% FBS (MD III) for another 3 days (ID 7). (Madsen et al., 2003). To define the effects of NDL PCBs on mature adipocytes, the cells were treated with PBC 101, 153 or 180 alone (1 μM) or in combination (1 μM) for two days (ID 9).

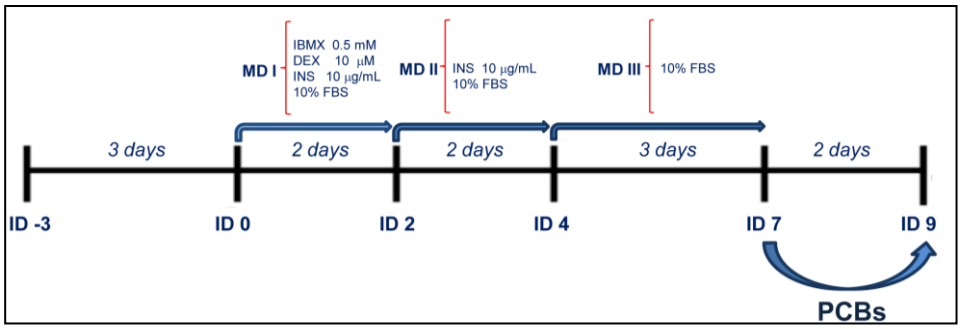


Fig. 20 Protocol for cell differentiation and treatment. The standard procedure of adipogenic differentiation is shown. 3T3-L1 cells were cultured to confluence (CF) for three days. Two days post-confluence (ID0) the cells were incubated in adipogenesis inducing medium (MD I) [DMEM containing 10 µM dexamethason (DEX), 0.5 mM isobutylmethylxanthine (IBMX), 10 µg/ml insulin (INS), and 10% FBS] for 2 days (ID2), then in adipogenesis maintaining medium (MD II) (DMEM containing 10 µg/ml INS and 10% FBS) for 2 days (ID4), followed by MD III medium (DMEM, 10% FBS) for another 3 days (ID7). To define the effects of PCBs on mature adipocytes, the cells were treated with PCBs from ID-7 to ID9.

6.3.3 Determination of cell viability

The viability of 3T3-L1 cells was determined by MTT test. Preadipocytes (8×10^3/well) were plated on 96-well microtiter plates to a final volume of 100 µL and were subjected to the same adipogenic differentiation above reported. At ID7 mature adipocytes were exposed to PCBs (10-100 nM and 1-10 µM). After two days, 25 µL of MTT (5 mg/mL) were added to each well and the cells were incubated for an additional 3 h. Thereafter, cells were lysed with 100 µL of a solution containing 50% (v/v) N,N-dimethylformamide, 20% (w/v) sodium dodecylsulphate (pH 4.5) to allow solubilization of dark blue crystals. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. 3T3-L1 viability in response to treatment with PCBs, alone or in combination, was calculated as % viable cells=(OD_{620} treated/OD_{620} control)×100.

6.3.4 Oil Red O staining

Preadipocytes were plated into 6-well plates (5×10^4cells/well). After adipogenic protocol and two days of exposure to PCBs, cell monolayers were rinsed twice with PBS and fixed in 10 % (vol/vol) formaldehyde in PBS for 60 min at room temperature. After washing with distilled water 2 times, fixed cells were stained with 1 mL/well of Oil Red O (ORO) working solution for 2 h. This solution was prepared as follows: 0.5 g of ORO was dissolved in 100 mL of absolute isopropanol allowed to stand overnight, and filtered through Whatman no. 1 filter paper. The filtrate was diluted with distilled water (6:4 vol/vol), left overnight at 4°C, and filtered twice. The staining solution was then discarded and the stained cells washed three times with distilled water. This is the Laughton method, slightly modified by Ramirez-Zacarias et al. (1992). To assess the lipid accumulation, 1 mL/well of isopropanol was added to each washed and dried stained well and the stained lipid was allowed to extract for 1 min. The extracted ORO absorbance was read spectrophotometrically at 510 nm.

6.3.5 Western blot analysis

After 48 h of incubation with PCBs, mature adipocytes were washed twice with ice cold PBS, harvested, and resuspended in 20 mM Tris-HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, leupeptin and trypsin inhibitor (10 μg/ml). After 1 h, cell lysates were obtained by centrifugation at 20,000 *g* for 15 min at 4°C. Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard.

Western blot analysis was performed as already reported loading 30 μg cell lysate proteins on 10% SDS–polyacrylamide. The filter was probed with anti-peroxisome

proliferator-activated receptor (PPAR)γ (1:1000), or anti-PTP1B, or anti-SOCS3 (dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-phosphoAMPKα and anti-AMPKα (dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA) or anti-phospho-STAT3 and anti-STAT3 (dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA) or anti-phosphoACC and anti-ACC (dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the β-actin protein (Sigma-Aldrich).

6.3.6 Real-time semi-quantitative PCR analysis

Total RNA, isolated from mature adipocytes treated with PCBs 101, 153 and 180, alone or in combination, was extracted by a modified method of Chomczynski & Sacchi (1987), using Trizol Reagent (Invitrogen Biotechnologies) in accordance with the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories) as previously described (Mattace Raso et al., 2014). The primer sequences are reported in Table 6.

Table 6 Real Time PCR Primer Sequences

Target gene	Forward primer (5' ⟶ 3')	Reverse primer (3' ⟶ 5')	Accession Number
Ob	5'-TCTCCGAGACCTCCTCCATCT-3'	3'-TTCCAGGACGCCATCCAG-5'	NM_008493.3
ObRb	5'-CCAGCACAATCCAATCACTAGTG-3'	3'-CGAATAGATGGATTATCGGGACA-5'	NM_146146.2
IL-6	5'-ACAAGTGGGAGGCTTAATTACACAT-3'	3'-TTGCCATTGCACAACTCTTTTC-5'	NM_031168.1
TNFα	5'-CATCTTCTCAAAACTCGAGTGACAA-3'	3'-TGGGAGTAGATAAGGTACAGCCC-5'	NM_013693.3
Rn18s	5'-CGCGGTTCTATTTTGTTGGT-3'	3'-AGTCGGCATCGTTTATGGTC-5'	NR_003278.3

The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol L^{-1} of each primer (Eurofins MWG Operon, Huntsville, AL) in a final volume of 25 µl. The relative expression of each studied mRNA was normalized to appropriate housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

6.3.7 Data analysis

Data are reported as mean ± standard error mean (S.E.M.) values of independent experiments, which were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test or Dunnet's test. When the P value was less than 0.05, the difference was considered statistically significant. Each analysis and calculations were performed by GraphPad Prism 5.

6.4 Experiments in chondrocytes

6.4.1 Reagents

Fetal bovine serum (FBS), MTT dye, human transferrin, sodium selenite, and PCB 101, 153 and 180 were obtained from Sigma (St. Louis MO, USA). PCBs were dissolved in dimethyl sulphoxide (DMSO) in a 10 mM stock solution. Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium, l-glutamine, antibiotics and trypsin-EDTA were purchased from Lonza (Verviers, Belgium).

6.4.2 Cell culture conditions and treatment

The murine chondrogenic cell line ATDC5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM–Ham's F-12 medium supplemented with 5% FBS, 10 µg/mL human transferrin, $3 \cdot 10^{-8}$ M sodium selenite, L-glutamine, and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin). The immortalized human juvenile costal chondrocyte cell line T/C-28a2 (a kind gift from Dr. Mary B. Goldring, Hospital for Special Surgery, NYC, USA) was culture in DMEM–Ham's F-12 medium supplemented with 10% FBS, L-glutamine, and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin).

6.4.3 Determination of cell viability

Cell viability was tested using the MTT reagent. Briefly, undifferentiated ATDC5 and human T/C-28a2 chondrocytes ($8 \cdot 10^3$/well) were seeded in 96-well plates. After overnight starvation, they were treated with DMSO (0.1%), PCBs (1-5-10 µM) for 24 and 48 h in serum free medium. Then, cells were incubated for 4 h with MTT reagent. After formazan salt was dissolved, absorbance was measured at 550 nm using microtiter enzyme-linked immunosorbent assay reader (Multiskan EX; Thermo Labsystems, Vantaa, Finland). Cells cultured with DMSO 0.1% were used to normalize cell viability status.

6.4.4 Apoptosis assay

FITC Annexin V Apoptosis Detection Kit II (Becton Dickinson) was used to identifying apoptotic or necrotic processes induced by PCBs. Briefly, ATDC5 cells ($1 \cdot 10^6$/well) were seeded in 6-well plates and treated with PCBs for 24 hours, at different

concentrations (1-5-10 μM). Next, cells were analysed according to the manufacturer's instructions of kit (BD Biosciences, New Jersey, USA).

6.4.5 Protein extraction and Western blot analysis

ATDC5 cells were plated at a density of $3 \cdot 10^5$ cells/well in 6-well plates. After 6 hours to allow adherence, cells were starved overnight in serum-free conditions. Cells were treated with different concentrations of PCBs (1, 5, 10 μM) for 3 and 24 hours at 37ºC in serum-free media. Control cells were treated with DMSO 0.1%. After that, cells were rapidly washed with ice-cold PBS and scraped in lysis buffer for protein extraction (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 0.5% Triton X-100, 1mM PMSF, protease inhibitor cocktail). Lysed cells were centrifuged at 14.000g for 20 min. Lysates from control or stimulated cells were collected and separated by SDS/PAGE on a 12% polyacrylamide gel. Proteins were subsequently transferred to a polyvinylidene difluoride transfer membrane (Immobilon-P transfer membrane, Millipore, MA) using a transfer semidry blot cell (BioRad Laboratories). The filters were incubated with antibody against cleaved caspase 3, caspase 3, Bcl-2 or Bax (Cell Signaling Technology, Danvers, MA, USA) and anti-phospho-p38 (Millipore, MA, USA). Immunoblots have been visualized with Immobilon Western Detection kit (Millipore, MA, USA) using horseradish peroxidase-labeled secondary antibody. To confirm equal loading in each sample, the membranes were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and re-blotted with anti-GAPDH antibody (Sigma Aldrich, St. Louis MO, USA). The images were captured and analyzed with an EC3 imaging system (UVP). Densitrometric

analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

6.4.6 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) levels, as marker of cell necrosis, were determined in cell supernatant. LDH converts L-lactate and nicotinamide adenine dinucleotide (NAD) to pyruvate and NADH, respectively. The rate of increase in absorbance of the reaction mixture at 340 nm, due to the formation of NADH, is proportional to the LDH activity. LDH was quantified with ADVIA® 1650 chemistry system. Thus, ATDC5 undifferentiated, ATDC5 mature and T/C-28a2 cells were cultured in 6-well plates ($1x10^6$ cells/well) and exposed to PCBs for 24 hours. Next, cell culture supernatants were harvested and LDH levels quantified. Hydrogen Peroxide (5 mM) was used as positive control.

6.4.7 Malondialdehyde measurement

ATDC5 cells were plated and starved as described for Western blot analysis. Then, cells were stimulated with PCB 101, 153 and 180 at increasing concentrations (1-5-10 µM). The concentration of pollutants was chosen on the basis of the experiments performed on cell viability. The final DMSO concentration in all well was 0.1 %. H_2O_2 (1 µM) treated cells were used as positive control.

After 24 hr exposure, MDA was evaluated in cell lysates, as a marker of lipid peroxidation, by the method described by Draper and Hadley (1990), which we have used previously (Mattace Raso et al., 2011). MDA standards were prepared by using 1,1,3,3-tetramethoxypropane in PBS. 900 µL of each sample were mixed with 2 mL of 10% TCA,

centrifuged at 1260 g for 10 min, followed by 1.3 mL of 0.5% TBA. After 20 min at 100 °C, fluorescence of the mixture was measured with a Perkin-Elmer spectrofluorimeter (model LS-5B; Perkin Elmer, Boston, MA, USA) at the wavelengths of 530 and 550 nm for excitation and emission, respectively. The MDA quantities were calculated by linear regression analysis of the standard curve. Values were expressed as µM MDA/mg proteins.

6.4.8 Total Antioxidant Status and Oxidative Stress Index determination

After 24 hr exposure to NDL-PCBs (1-5-10 µM), Total Antioxidant Status (TAS) and Oxidative Stress Index (OSI) were evaluated in ATDC5 cell lysates, by using two proper kits (Rel Assay Diagnostics®), according to manufacturer's protocols.

TAS was measured with a kit, wherein antioxidants in the sample reduced the dark blue-green colored 2,2'-azinobis-(3-ethylbenzthiazoline sulphonate) (ABTS) radical to colourless reduced ABTS form, which is relatively stable and can be measured at 600 nm. Antioxidants in the sample caused suppression of the production of this colour to a degree that was proportional to their concentration. The assay was calibrated with a stable antioxidant standard solution which is traditionally named as Trolox Equivalent that is vitamin E analogue. TAS, determined by using this automated measurement method developed by Erel (Erel, 2004), was finally expressed as mmol Trolox Equiv./L. Conversely, Total Oxidant Status (TOS) was determined using a proper kit, following a method previously described by Erel (Erel, 2005), and was expressed as μmol H_2O_2 Equiv./L. Finally, the OSI was calculated from the TOS and TAS values: OSI = [(TOS, μmol H_2O_2 Equiv./L)/(TAS, μmol Trolox Equiv./L)] × 100 (Cekic et al., 2014; Aycicek et al., 2005).

6.4.9 Statistical analysis

Data are reported as the mean ± SEM of at least 3 independent experiments, each with at least 3 independent observations. Statistical analysis was performed using one-way ANOVA test followed by the Student-Newman-Keuls test or Bonferroni multiple comparison test using the Prism computerized package (GraphPad Software V.5, La Jolla, CA). P-values less than 0.05 were considered significant.

Chapter 4

7. RESULTS

7.1 PCBs and OCPs concentrations in Mediterranean mussel (*Mytilus Galloprovincialis*) and razor clam (*Ensis Siliqua*) from Castelvolturno, Campania Region (Italy)

The mean Length and Weight of the specimens were: 5.75 cm and 16.03 g for mussels and 12.4 cm and 15.9 g for clams. The mean lipid contents were 2.46 % and 2.59 % for mussels and clams, respectively.

For both species, PCBs were the most abundant pollutants followed by DDTs, Dieldrin and HCB; the results are showed in Fig. 21 and Table 5.

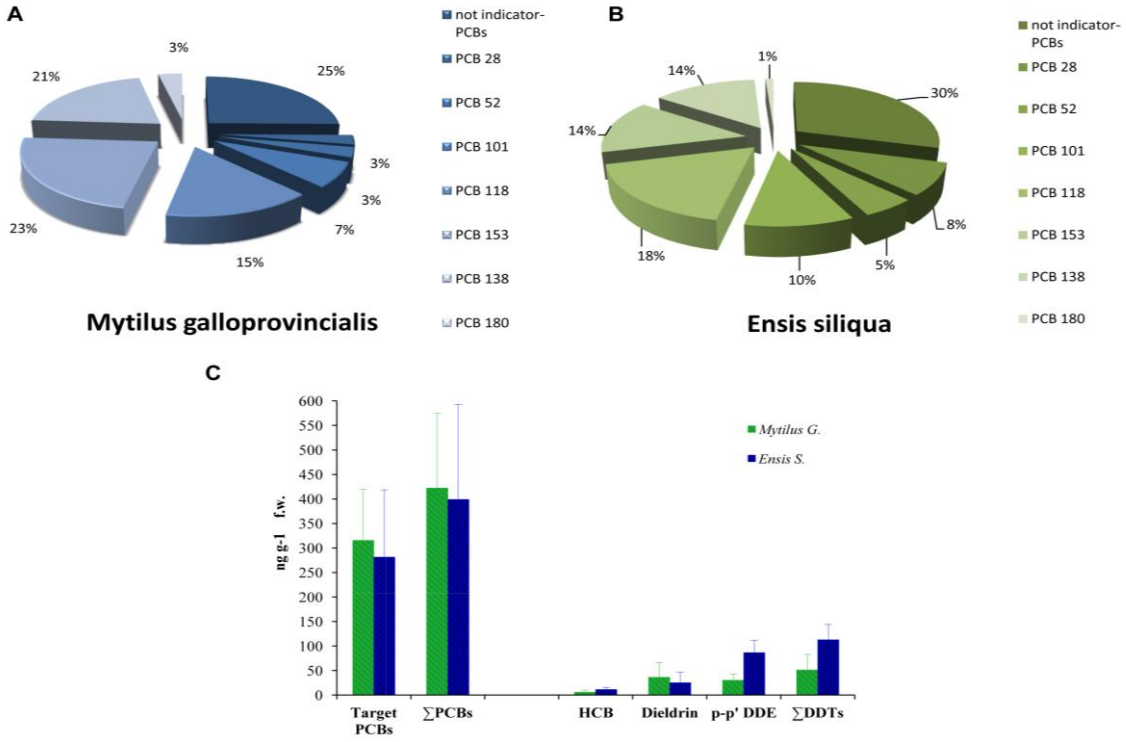


Fig. 21 Concentrations of PCBs and OCPs in the edible tissues of two bivalve species (Mytilus Galloprovincialis and Ensis Siliqua) from the coastal area fo Castelvolturno (Caserta). Panel A shows the concentrations (% of ∑PCBs) of the PCBs analyzed in Mediterranean mussels; Panel B shows the concentrations (%of ∑PCBs) of the PCBs analyzed in razor clams and Panel B shows the concentrations (ng g^{-1} on fat weight) of PCBs and OCPs in both the analyzed species.

Table 5 Median, Mean ± SD, range of organochlorine concentrations (ng g^{-1} on LW and WW) in edible tissues of mussels and clams from Castelvolturno; % of total mussel and clam sample units contaminated by each compound and difference between the means on LW are also presented.

	Median Mean ± SD *(Range)*						
	Mediterranean mussels			Razor clams			Mean difference on LW
	%	LW	WW	%	LW	WW	
OCPs (ng g^{-1})							
HCB	87.5	**5.23** 5.67 ± 4.37 *(nd[a] – 14.38)*	**0.16** 0.14 ± 0.10 *(nd. – 0.38)*	100	**11.14** 11.52 ± 3.99 *(4.49 – 19.28)*	**0.28** 0.27 ± 0.05 *(0.19 – 0.35)*	5.85
Dieldrin	92.5	**33.01** 36.33 ± 30.09 *(nd – 142.41)*	**0.73** 0.76 ± 0.60 *(nd. – 3.00)*	100	**18.39** 25.46 ± 21.28 *(4.34 – 83.34)*	**0.46** 0.58 ± 0.38 *(0.14 – 1.53)*	10.87
p,p'-DDE	100	**31.00** 30.27 ± 12.26 *(4.36 – 57.56)*	**0.70** 0.70 ± 0.29 *(0.10 – 1.26)*	100	**87.60** 86.67 ± 25.32 *(44.39 – 154.84)*	**1.88** 2.12 ± 0.62 *(1.44 – 3.76)*	56.4**
p,p'-DDD	90	**10.78** 12.30 ± 8.64 *(nd – 32.90)*	**0.26** 0.27 ± 0.18 *(nd. – 0.77)*	100	**23.12** 24.47 ± 7.77 *(12.11 – 41.25)*	**0.53** 0.59 ± 0.17 *(0.35 – 0.99)*	12.17
p,p'-DDT	55	**4.25** 8.64 ± 21.16 *(nd – 130.07)*	**0.08** 0.15 ± 0.30 *(nd – 1.76)*	80	**1.93** 1.85 ± 1.48 *(nd – 5.30)*	**0.05** 0.05 ± 0.03 *(nd – 0.11)*	6.79**
ΣDDTs	100	**45.82** 51.21 ± 31.95 *(12.88 – 217.69)*	**1.08** 1.12 ± 0.47 *(0.45 – 2.94)*	100	**112.29** 112.99 ± 31.05 *(58.15 – 196.09)*	**2.55** 2.76 ± 0.75 *(2.04 – 4.82)*	61.78
PCBs (ng g^{-1})							
PCB 28	75	**11.42** 11.26 ± 9.41 *(nd. – 42.91)*	**0.26** 0.27 ± 0.20 *(nd. – 0.65)*	100	**35.01** 33.47 ± 12.86 *(15.30 – 56.67)*	**0.73** 0.78 ± 0.20 *(0.51 – 1.23)*	22.21
PCB 52	75	**11.34** 12.00 ± 9.75 *(nd – 36.09)*	**0.31** 0.29 ± 0.21 *(nd – 0.82)*	100	**16.58** 20.36 ± 9.92 *(8.00 – 37.17)*	**0.39** 0.47 ± 0.18 *(0.25 – 0.95)*	8.36
PCB 74	42.5	**0.00** 2.70 ± 4.03 *(nd. – 14.55)*	**0.00** 0.06 ± 0.08 *(nd. – 0.28)*	53.33	**0.32** 2.12 ± 2.85 *(nd. – 8.20)*	**0.01** 0.05 ± 0.06 *(nd – 0.15)*	0.58
PCB 66	37.5	**0.00** 5.87 ± 12.33 *(nd – 59.57)*	**0.00** 0.13 ± 0.28 *(nd – 1.40)*	53.33	**3.76** 11.53 ± 14.37 *(nd. – 35.39)*	**0.07** 0.25 ± 0.33 *(nd. – 1.06)*	5.66
PCB 101	90	**29.99** 29.84 ± 18.04 *(nd. – 95.81)*	**0.72** 0.68 ± 0.34 *(nd. – 1.49)*	100	**31.80** 40.52 ± 25.57 *(17.84 – 101.12)*	**0.71** 0.97 ± 0.59 *(0.59 – 2.73)*	10.68
PCB 99	72.5	**13.38** 19.86 ± 34.64 *(nd – 215.68)*	**0.36** 0.46 ± 0.71 *(nd – 4.18)*	93.33	**22.80** 25.63 ± 19.63 *(nd – 74.72)*	**0.56** 0.65 ± 0.46 *(nd – 1.64)*	5.77
PCB 118	97.5	**67.48** 64.62 ± 26.33 *(nd – 109.16)*	**1.42** 1.43 ± 0.57 *(nd – 3.11)*	100	**51.36** 70.40 ± 39.81 *(30.00 – 170.81)*	**1.32** 1.70 ± 0.91 *(0.96 – 4.22)*	5.78*
PCB 105+146	70	**14.20** 15.23 ± 13.70 *(nd – 43.75)*	**0.37** 0.38 ± 0.33 *(nd – 1.21)*	100	**22.09** 27.29 ± 20.80 *(10.82 – 80.84)*	**0.45** 0.66 ± 0.47 *(0.20 – 1.85)*	12.06
PCB 153	100	**103.90** 97.13 ± 32.88 *(22.96 – 161.88)*	**2.10** 2.19 ± 0.71 *(0.45 – 4.72)*	100	**46.58** 56.88 ± 27.28 *(25.33 – 118.46)*	**1.15** 1.36 ± 0.58 *(0.84 – 3.07)*	40.25
PCB 138	100	**88.68** 86.60 ± 31.50 *(26.49 – 171.35)*	**1.83** 1.90 ± 0.45 *(1.15 – 3.25)*	100	**42.96** 55.59 ± 31.62 *(24.53 – 126.07)*	**1.08** 1.34 ± 0.73 *(0.72 – 3.50)*	31.01
PCB 128+187	97.5	**29.85** 29.02 ± 15.59 *(nd – 67.36)*	**0.64** 0.67 ± 0.37 *(nd. – 1.76)*	100	**16.84** 19.60 ± 13.16 *(3.04 – 52.14)*	**0.44** 0.47 ± 0.28 *(0.06 – 0.97)*	9.42
PCB 183	97.5	**13.00** 13.71 ± 7.39 *(nd – 33.36)*	**0.34** 0.33 ± 0.19 *(nd. – 0.79)*	86.66	**2.75** 3.15 ± 2.37 *(nd. – 7.75)*	**0.07** 0.07 ± 0.05 *(nd – 0.15)*	10.56
PCB 177	100	**11.99** 11.72 ± 4.40 *(1.13 – 20.99)*	**0.25** 0.26 ± 0.07 *(0.02 – 0.46)*	100	**11.87** 12.54 ± 6.35 *(5.44 – 24.83)*	**0.32** 0.33 ± 0.20 *(0.10 – 0.87)*	0.82
PCB 180	100	**10.41** 14.27 ± 20.53 *(3.09 – 138.50)*	**0.23** 0.30 ± 0.31 *(0.13 – 2.12)*	100	**4.19** 4.27 ± 1.68 *(1.45 – 7.08)*	**0.10** 0.10 ± 0.03 *(0.05 – 0.18)*	10**
PCB 170	95	**5.92** 6.50 ± 4.67 *(nd – 20.69)*	**0.12** 0.15 ± 0.13 *(nd – 0.65)*	73.33	**1.71** 2.36 ± 2.56 *(nd – 8.33)*	**0.04** 0.06 ± 0.05 *(nd – 0.15)*	4.14
PCB 201	22.5	**0.00** 0.55 ± 2.06 *(nd. – 12.68)*	**0.00** 0.01 ± 0.05 *(nd. – 0.03)*	33.33	**0.00** 0.96 ± 2.15 *(nd. – 7.78)*	**0.00** 0.02 ± 0.03 *(nd. – 0.12)*	0.41
PCB 196	55	**0.30** 0.85 ± 1.21 *(nd – 5.36)*	**0.01** 0.02 ± 0.03 *(nd. – 0.11)*	100	**1.69** 1.91 ± 0.87 *(0.84 – 3.95)*	**0.05** 0.05 ± 0.02 *(0.02 – 0.10)*	1.06
PCB 194	40	**0.00** 0.44 ± 0.62 *(nd. – 2.31)*	**0.00** 0.01 ± 0.02 *(nd. – 0.06)*	100	**10.73** 10.76 ± 4.86 *(4.79 – 22.97)*	**0.28** 0.26 ± 0.10 *(0.09 – 0.40)*	10.32
Indicator-PCBs	100	**333.85** 315.72 ± 103.63 *(112.25 – 549.72)*	**6.73** 7.06 ± 2.03 *(2.93 – 14.39)*	100	**222.09** 281.48 ± 137.14 *(123.46 – 592.35)*	**5.50** 6.72 ± 3.01 *(4.15 – 15.55)*	34.24
ΣPCBs	100	**429.48** 422.19 ± 152.62 *(146.86 – 879.61)*	**8.92** 9.54 ± 3.37 *(3.66 – 21.65)*	100	**326.87** 399.33 ± 193.33 *(175.41 – 865.99)*	**7.70** 9.59 ± 4.29 *(5.74 – 21.37)*	22.86

[a] Not detectable
*P<0.05; **P<0.001

7.1.1 PCBs

PCBs were found in all sample units. Overall the statistical analysis concerning the difference in PCBs accumulation across species evidenced a slight greater presence in mussels. The concentration levels of the seven indicator congeners, summed up for each sample unit and then averaged across the entire sample, amounted to about 75% and 70% of all PCBs quantities in mussels and clams, respectively. In particular, the indicator penta- and hexa-chlorinated PCBs (IUPAC nos. 101, 118, 138, 153) accounted for 66% and 56% of the total, respectively, for mussels and clams; the PCB 153 was the dominant congener for the mussels while in clams PCB 118 showed the highest concentration. Among the seven congeners a strong significant difference was observed for PCB 180 (P= 0.0001) which was higher in mussels; the converse was true for PCB 118 (P= 0.0212). The PCBs accumulation profile in razor clams was also characterised by relative high contributions of tri-, tetra-, and penta-chlorinated biphenyls 28, 52, 66, 74, 99 and 101 (which amounted to 33.70% of ∑PCBs) and small concentrations of the octa-chlorobiphenyls 201, 196 and 194 (which amounted to 3.75% of ∑PCBs). The mussels showed lower presence of the PCBs 28, 52, 66, 74, 99 and 101 (18.44% of ∑PCBs) compared to the clams, and almost the absence of the octa-chlorobiphenyls 201, 196 and 194 (0.47% of ∑PCBs). Difference between species was statistically significant for the first group of congeners at 5% level (P= 0.021) and extremely significant for the second one (P= 0.0001).

7.1.2 OCPs

In the clams the dominant pesticide, revealed in all the sample units, was p,p'-DDE, followed by p,p'-DDD and p,p'-DDT; in the mussels the levels of OCPs were as

follows: Dieldrin>p,p'-DDE>p,p'-DDD>HCB>p,p'-DDT. Dieldrin was detected in 92.5% of the mussel sample and in all clams suggesting a recent use of such pesticide in the fields along the coastal area.

The p,p'-DDE median concentration was higher than that of the p,p'-DDT for both species (31 vs. 4.25 and 87.6 vs. 1.93 ng g^{-1} LW, respectively, for mussels and clams). As DDT is metabolised to DDE, the DDE/DDT ratio is suggested as an useful index to assess the chronology of DDT entering the ecosystems; in particular, a value greater than one signals a prevalently past use of the pesticide (Bordajandi et al., 2003). When the averages of DDE and DDT concentrations across mussels or clams are used to construct such ratio, it follows a value of 3.5 for the former and 46.8 for the latter. This evidence accords with the high persistence of the OCPs in the sediments and thus with the bioaccumulation of the metabolite in the benthic species.

HCB was found in 80% of the mussels and in all clams analysed, although with low residue levels. The wide presence may be ascribed not only to its previous use as a fungicide treatment for seeds but also to the fact that it is a by-product in the manufacturing processes of various chlorine-containing chemicals and an impurity in several chemical processes [International Programme on Chemical Safety (IPCS), 1997].

About OCPs, overall the residue levels for HCB and DDTs, but p,p'-DDT, were higher in razor clams than mussels with a highly significant difference for p,p'-DDE (P= 0.0002). Dieldrin and p,p'-DDT, instead, were lower respect to *M. galloprovincialis*, with a very significant difference for the last compound (P= 0.0001).

7.1.3 Comparison with previous studies

Table 6 compares our results with those relative to Mediterranean mussel and a similar species, the *Mytilus edulis*, reported by other authors. A systematic comparison

regarding the *Ensis siliqua* misses because, to the best of our knowledge, the only paper considering such species is by Carro et al. (2006), who reported residue levels for specimens collected in Galicia (Spain) quite similar to those we did.

Table 6. Organochlorine concentrations (mean± S.D. expressed as ng g^{-1} on lipid weight) in *Mytilus* spp. from different parts of the world.

Location	Species	HCB	Dieldrin	DDTs	Indicator-PCBs	References
Castelvolturno - Tyrrenian Sea, Italy	*M. galloprovincialis*	5.67 ± 4.37	36.33 ± 30.09	51.21 ± 31.95	315.72 ± 103.63	Present study
Gulf of Naples - Tyrrenian Sea, Italy	*M. galloprovincialis*	11.71 ± 8.53	Na [a]	113.62 ± 116.91	2079.20 ± 2153.88	Ferrante et al., 2007
Gulf of Naples - Tyrrenian Sea, Italy	*M. galloprovincialis*	12.45 ± 10.32	Na	177.81 ± 118.99	1519.48 ± 417.22	Naso et al., 2005
Central Adriatic Sea, Italy [b]	*M. galloprovincialis*	Na	Na	209.68; 159.24 [c, d]	336.79; 752.34 [c]	Perugini et al., 2004
Central Adriatic Sea, Italy [b]	*M. galloprovincialis*	Na	Na	Na	5.48 [c, e]	Piersanti et al., 2006
Adriatic Sea, Italy [f]	*M. galloprovincialis*	Na	Na	187.5; 135.71; 114.29 [c, g]	1156.25; 94.28; 425 [c, h]	Bayarri et al., 2001
Faro lake, Italy	*M. galloprovincialis*	Na	Na	< 0.05 - 275 [g, n]	< 0.8 [i]	Licata et al., 2004
North-West Mediterranean coast, France and Italy	*M. galloprovincialis*	0 - 1.9 [l, o]	0 - 36 [l, o]	15.55 - 568.58 [l, o]	50 – 4057 [l, m, o]	Villeneuve et al., 1999
Thau lagoon, France [n]	*M. galloprovincialis*	Na	Na	Na	9.836; 21.637; 38.699 [c]	Castro-Jiménez et al., 2008
Ebro delta, Spain	*M. galloprovincialis*	<0.1 - 0.2 [e, o]	Na	1336 [c] (240 - 6838) [o]	953 [c] (264 - 3329) [o]	Solé et al., 2000
Catalonian coasts, Spain	*M. galloprovincialis*	Na	Na	Na	14.02 [c, e] (2.19 - 51.1) [e, o]	Porte and Albaiges, 1993
Galician coasts, Spain	*M. galloprovincialis*	Na	Na	Na	0.62 - 107.5 [c, o, p]	Carro et al., 2010
Mokpo bay, Korea	*M. galloprovincialis*	Na	Na	Na	<1 – 15 [l, o]	Namiesnik et al., 2008
Asian coastal waters [q]	*M. galloprovincialis and M. edulis*	111.6; 2.3; 8.2 [c]	Na	9366; 150; 270 [c]	269.8; 170; 3000 [c, r]	Monirith et al., 2003
Arcachon Bay, France	*M. edulis*	Na	Na	4.32 [c, s]	6.28 [c, t]	Thompson et al., 1999
South-West Baltic Sea, Germany [u]	*M. edulis*	0 - 5.2 [l, o]	Na	5.1 - 88.3 [l, o]	29.2 - 487.1 [l, o, v]	Lee et al., 1996
Norway	*M. edulis*	2.8 ± 1.8	Na	20.9 ± 10.1	79.5 ± 59.3	Green and Knutzen, 2003
Greenland - western coast	*M. edulis*	0.069 ± 0.017 [c]	Na	0.49 ± 0.26 [c]	0.95 ± 0.29 [c, p]	Cleeman et al., 2000
Usuk, southern Greenland	*M. edulis*	4.07 [c]	Na	22.4 [c]	28.0 [c, z]	Glasius et al., 2005

[a] Not available.
[b] Samples from 2 sites.
[c] Mean.
[d] Sum of p,p'-DDTs and o,p'-DDT.
[e] Wet weight.
[f] Data referred to North, Centre and South Adriatic Sea, respectively.
[g] p,p'-DDE.
[h] Sum of indicator-PCBs and PCB 163.
[i] Aroclor 1232 series.
[l] Dry weight.
[m] Sum of Aroclor 1254 and PCBs nos. 52, 101, 138 and 153.
[n] Samples from 3 sites.
[o] Range.
[p] Sum of indicator-PCBs and PCBs nos. 31, 105 and 156.
[q] Values referred, respectively, to China, Korea and Japan.
[r] Sum of PCB congeners of a mixture of Aroclor (1016:1242:1254:1260).
[s] Sum of p,p'-DDT and o,p'-DDT.
[t] Sum of indicator-PCBs and PCBs nos. 18, 44, 66, 95, 87, 105, 128, 126, 187, 170, 195, 206 and 209.
[u] Samples from 10 sites.
[v] Sum of PCBs nos. 52, 151, 149, 118, 153, 138, 187, 183 and 180.
[z] Sum of indicator-PCBs and PCBs nos. 31, 105, 128, 149, 156 and 170.

Our measures of DDTs are rather comparable to those reported for other Mediterranean and non Mediterranean areas (Bayarri et al., 2001; Licata et al., 2004; Thompson et al., 1999), the two exceptions being related to mussels from Ebro delta in Spain (Solé et al., 2000) and from Asian coastal waters (Monirith et al., 2003), characterised by substantially higher concentrations of DDTs. Measures of PCBs are, in general, lower or similar to other Mediterranean and non Mediterranean regions (Bayarri et al., 2001; Carro et al., 2010; Castro-Jiménez et al., 2008; Lee et al., 1996; Monirith et al., 2003; Namiesnik et al., 2008; Perugini et al., 2004; Piersanti et al., 2006; Solé et al., 2000; Villeneuve et al., 1999). Surprisingly, PCB levels resulted higher than those relative to the coastal areas of Norway and Greenland characterized by high anthropogenic impact (Cleeman et al., 2000; Glasius et al., 2005; Green and Knutzen, 2003).

PCBs mean concentrations, detected in Mediterranean mussels, are lower than those found few years ago in the Gulf of Naples (Campania region, Italy) by Ferrante et al. (2007) and Naso et al. (2005) — $P<0.001$ and $P<0.05$, respectively; analogously for DDTs — $P<0.001$ and $P<0.05$ with respect to Naso et al. and Ferrante et al. (Table 6). This might suggest a decreasing time trend of OCs concentrations. An alternative explanation rests instead on the different sampling areas of the present and previous studies, which implies different sources of contaminants. In fact, the area of Castelvolturno is less affected by urban and industrial activities than the Gulf of Naples. Further, since body burden of organochlorines tends to reduce through reproduction (Bergen et al., 2001), another possible explanation may be a seasonality effect: regarding the present study, sampling was immediately post-ovodeposition while, on the contrary, it was pre-ovodeposition in the other two papers. By the way, note that the observed trend accords with data collected in the last decades about PCBs concentrations in biota from freshwater environments,

which show that PCBs levels have decreased at various rates or have approached a steady state condition (Smith, 2000).

7.1.4 Risk evaluation

It has been shown that OCs in marine organisms affect many biological/physiological processes including lipid metabolism and many endocrine system functions (Letcher et al., 2010). Studies about invertebrates are however scarce. PCBs affect the immune system of bivalves (Liu et al., 2009) and, analogously to OCPs, are responsible for genotoxicity (Binelli et al., 2008) as well as for damages to reproduction, mainly inducing degeneration and delayed maturation of oocytes (Binelli et al., 2004; Lehmann et al., 2007). The present study suggests that acute effects of OCs seem to be unlikely; chronic damages, such as effects on immune system and on reproduction, may be instead realistic.

For the human hazard evaluation, in Italy the maximum residue limits (MRLs) of OCPs are established for fish and aquatic products by the Decreto Ministeriale of 27 August 2004. All mussel sample unit, but one, are characterised by a lipid percentage less than 5% and are in group 1 of the 4th enclosure, with MRL 0.050, 0.010, 0.005 μg g^{-1} WW respectively for DDTs, HCB and Dieldrin. All the analysed samples are below these limits, according to the organochlorine pesticides ban in agricultural uses. Therefore, the data suggest that the organochlorine pesticides in bivalves caught in the area of Castelvolturno are unlikely to cause a significant health hazard for the consumers. Regarding PCBs, the EU legislation has not fixed an MRL for the seven indicator-PCBs relative to fish, shellfish or crustaceans. At the moment, it still applies the limit of 200 ng g^{-1} LW for the sum of the concentrations of the seven indicator congeners for terrestrial edible class of food (EU

Commission Decision 1999/788). The data collected evidence that the indicator-PCBs concentrations exceed the above mentioned limit both in mussels and clams in a percentage of cases, respectively, of 82.5% and 73.3%.

Since pollutants such as OCs accumulate in organisms through the food chain, the diet is considered the main source of human exposure with an important role held by animal fats and sea products in many western diets (Bayarri et al., 2001; Fattore et al., 2008). Corsolini et al. (1995) reported that PCBs 118, 138, 153 were among the most abundant PCB congeners found in human adipose tissue from Italian surgical patients. In the present study, analogously to other authors (Carro et al., 2010; Castro-Jimenez et al., 2008), we show that those congeners are the predominant ones in the analysed bivalves.

However, since dietary habits vary greatly among populations and different residue levels of OCs emerge across geographical areas, the foods through which one can be exposed to these compounds as well as the exposure levels themselves may differ across populations.

In 2009, consumption of bivalve molluscs in the Italian macro region including Abruzzo, Molise, Campania and Puglia, was 37% of total consumption in Italy [Istituto di Servizi per il Mercato Agricolo Alimentare (ISMEA)-AcNielsen, 2009], the highest percentage in Italy compared to the other macro regions. Although the species analysed showed residue levels lower than those observed in other sea products from Campania region, their relevant presence in the diet and frequency of consumption may represent a critical factor in the risk evaluation.

7.2 Immune innate response results compromised in macrophages exposed to NDL-PCBs

7.2.1 Effects of PCBs in presence or not of LPS on J774A.1 cell viability

The cytotoxicity of J774A.1 cells incubated for 24 h with increasing concentrations of all PCBs examined (100 nM–10 µM) alone or in combination with LPS (10 ng/mL) was evaluated by MTT assay (Figs. 22A-C).

All NDL-PCBs tested induced a concentration dependent cytotoxic effect. When comparing optical density values of control and PCB-treated cells, the cytotoxic effect of the pollutants was significant at the highest concentration used (10 µM, P<0.05).

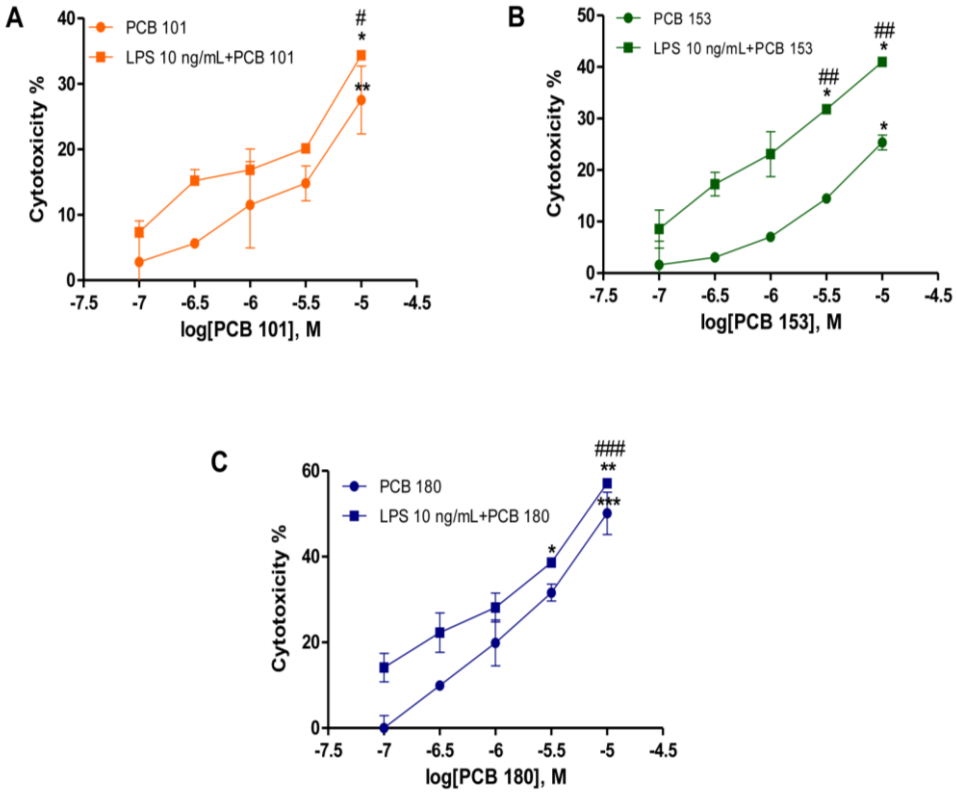


Fig. 22 Cytotoxic effects of NDL-PCBs 101, 153 and 180 alone or in combination with LPS on macrophage J774A.1 cells. Cells were treated for 24 h with NDL-PCBs tested at increasing concentrations (100 nM-10 µM) in presence of LPS (10 ng/mL). Cytotoxicity was then evaluated as described in Material and Methods section. Each per cent value is the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 vs control cells; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 vs LPS incubated cells.

Among the pollutants assayed, the PCB 180 was the most cytotoxic because it determined a reduction of J774A.1 viability of 45% at 10 µM concentration and it showed an IC_{50} value of $1.41 \cdot 10^{-5}$ M when used alone without LPS challenge. In fact the IC_{50} values of PCB 101 and PCB 153 were $1.91 \cdot 10^{-3}$ M and $2.19 \cdot 10^{-3}$ M respectively, and moreover, they raised a reduction of cell viability of only the 27-22% when used alone at the highest concentration (Table 7).

PCBs were less cytotoxic at the same concentrations after 4 h of incubation (data not shown). Interestingly, the concentration of 100 nM, used for the further experiments here shown, was not significantly cytotoxic for all NDL-PCBs tested. Afterwards, when cells were co-stimulated for 24 h with LPS, at concentration that alone did not modify the cellular viability, increased toxic effects of these pollutants were evidenced with a $4.57 \cdot 10^{-4}$ M, $4.79 \cdot 10^{-5}$ M and $1.15 \cdot 10^{-5}$ M IC_{50} values for PCB 101, 153 and 180 respectively. As reported in the Figs. 22A-C these effects were concentration-dependent and strongly significant at 10 µM ($P<0.05$).

Table 7 Effects of NDL-PCBs 101, 153 and 180, alone (100 nM-10 µM) and in combination with LPS (10 ng/mL), on J774A.1murine macrophage cell line viability. The viability of control cells was designated as 100% and results were expressed as the concentration of NDL-PCBs able to induce the 50% of mortality in untreated or LPS-treated macrophages (IC_{50}). Results are expressed as mean±s.e.m. from at least three independent experiments.

CELL TREATMENT	IC_{50} value [M]
PCB 101 (100 nM-10 µM)	$1.91*10^{-3}$
PCB 153 (100 nM-10 µM)	$2.19*10^{-3}$
PCB 180 (100 nM-10 µM)	$1.41*10^{-5}$
PCB 101 (100 nM-10 µM)+ LPS 10 ng/mL	$4.57*10^{-4}$
PCB 153 (100 nM-10 µM)+ LPS 10 ng/mL	$4.79*10^{-5}$
PCB 180 (100 nM-10 µM)+ LPS 10 ng/mL	$1.15*10^{-5}$

7.2.2 PCBs, alone or in combination, reduce mRNA levels of chemokine and pro-inflammatory cytokine in LPS-activated J774A.1 macrophages

As reported by Huang et al. (2012), the pattern of cytokine expression after LPS challenge depends on exposure time and LPS concentration. Its modulation is related to the different regulation of TLR-4 at transcriptional level. As well known, IL-6 and TNFα, two pro-inflammatory cytokines, and the chemokine MCP-1 play a key role in different transduction pathways of immune innate response. As shown in Fig. 23A-F, LPS (10 ng/mL) induced significant increases in IL-6, TNF-α and MCP-1 mRNA levels in J774A.1 cells after 4 hours of exposure.

The hexa- and hepta-chlorinated congeners inhibited the LPS-induced transcription of pro-inflammatory cytokines and chemokine. In particular, both PCB 153 and PCB 180 significantly suppressed LPS-induced IL-6 expression (Fig. 23A; $P<0.01$ and $P<0.001$, respectively).

Interestingly, PCB 153 reduced LPS-induced MCP-1 mRNA level (Fig. 23C; $P<0.05$) whilst PCB 180 suppressed LPS-induced TNF-α expression (Fig. 23B; $P<0.05$). Conversely, the penta-chlorinated congener, PCB 101, was the less immunotoxic among the PCBs analyzed.

It is noteworthy that all the combinations of the aforesaid NDL-PCBs showed a synergist effect, since they significantly reduced LPS-induced cytokines expression (Fig. 23D, E and F).

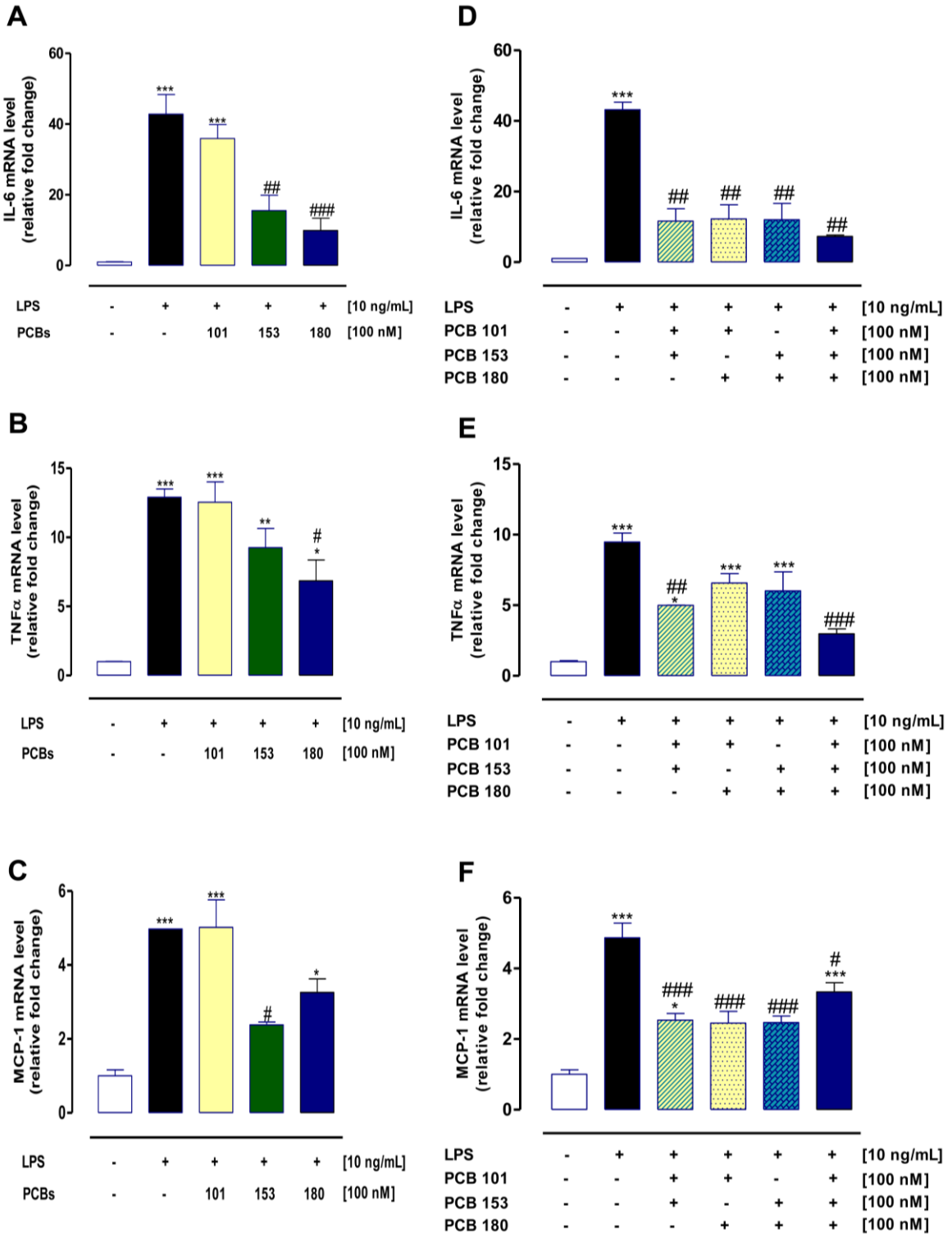


Fig. 23 Effects of NDL-PCBs 101, 153 and 180, alone or in combination, on LPS-induced mRNA expressions of IL-6, TNFα and MCP-1. J774A.1 macrophages were exposed to NDL-PCBs at 100 nM, both alone and mixed, in presence of LPS (10 ng/mL) for 4 hours. After that, cells were lysed for collection of RNA performed as described in Meterials and Methods section. The mRNA expression levels of cytokines were analyzed by real-time PCR. Panels A and B: IL-6 levels. Panels C and D: TNF-α levels. Panels E and F: MCP-1 levels. Data are means ± S.E.M. of 3 independent experiments. *P< 0.05, **P<0.01 and ***P<0.001 vs untreated cells; $^{\#}$P < 0.05; $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 vs LPS treated cells.

7.2.3 Effects of PCBs, alone or in combination, on LPS-induced COX-2 expression

In order to asses if NDL-PCBs influence LPS-induced COX-2 expression we evaluated the protein level in cellular lysates from murine macrophage cell line J774A1 exposed to these pollutants (100 nM), alone (Fig. 25A) or in combination (Fig. 25B), in presence of LPS (10 ng/mL). A marked increase in the expression of this pro-inflammatory enzyme in cellular lysates was observed at 24 h after LPS challenge ($P<0.001$, vs untreated cells). When macrophages were exposed to PCBs alone only a trend of reduction of LPS-induced expression of COX-2 was shown (Fig. 25A); but, interestingly, when PCBs were added to cells in combination, two by two or all together, a strongly reduction of bacterial endotoxin-induced COX-2 protein level was evidenced ($P<0.001$, vs LPS-stimulated cells; Fig. 25B).

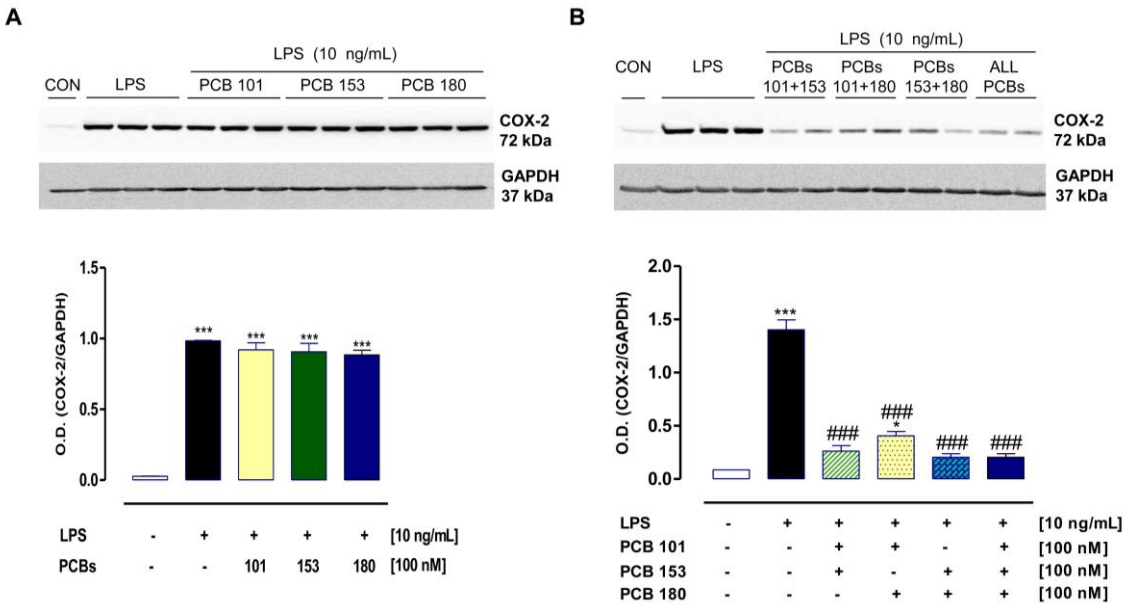


Fig. 25 Effects of NDL-PCBs 101, 153 and 180, alone (Panel A) or in association (Panel B), in presence of LPS stimuli, on COX-2 protein expression. Lysates were obtained from control and 24 h-treated cells with NDL-PCBs at 100 nM, alone or differently associated, in presence of LPS (10 ng/mL). A representative immunoblot is shown. Densitometric analysis of protein bands was performed on three separate experiments. GAPDH protein immunoblot was performed to ensure equal sample loading. *$P < 0.05$, ***$P < 0.001$ vs control cells; $^{###}P < 0.001$ vs LPS treated cells.

7.2.4 Effects of PCBs, alone or in combination, on LPS-induced iNOS expression and NO^{-2} production

iNOS expression was evaluated by Western blot analysis to determine the modulatory effect of NDL-PCBs, alone or in combination, flowing LPS on iNOS expression. We investigated the effects of NDL-PCBs, alone or in combination, on NO^{2-} production in LPS-stimulated J774A.1 cells, since macrophages play a pivotal role in a host's defense against microbial infection through the production of variety of chemicals including NO^{2-} (Billack, 2006). The bacterial endotoxin showed a significant increase in iNOS expression similarly to that shown in COX-2 (Figs. 26A and 26B). When cells were co-exposed to LPS and the evaluated pollutants, only the PCB 180 alone induced a significant decrease of iNOS expression compared to that of LPS-stimulated cells ($P<0.05$, Fig. 26A). Conversely, all the combination of NDL-PCBs, at the same concentration used alone, markedly reduced iNOS expression ($P<0.001$ vs LPS-treated cells; Fig. 26B).

Accordingly, significantly increased production of nitrite compared to that of untreated control cells was observed ($P<0.001$; Figs. 27A and 27B). This induction of nitrite generated by LPS was inhibited only by PCB 180 and by all PCB combinations, confirming the synergistic effect (Fig. 27A and B), without any effects on cell viability (Figs. 23A-C).

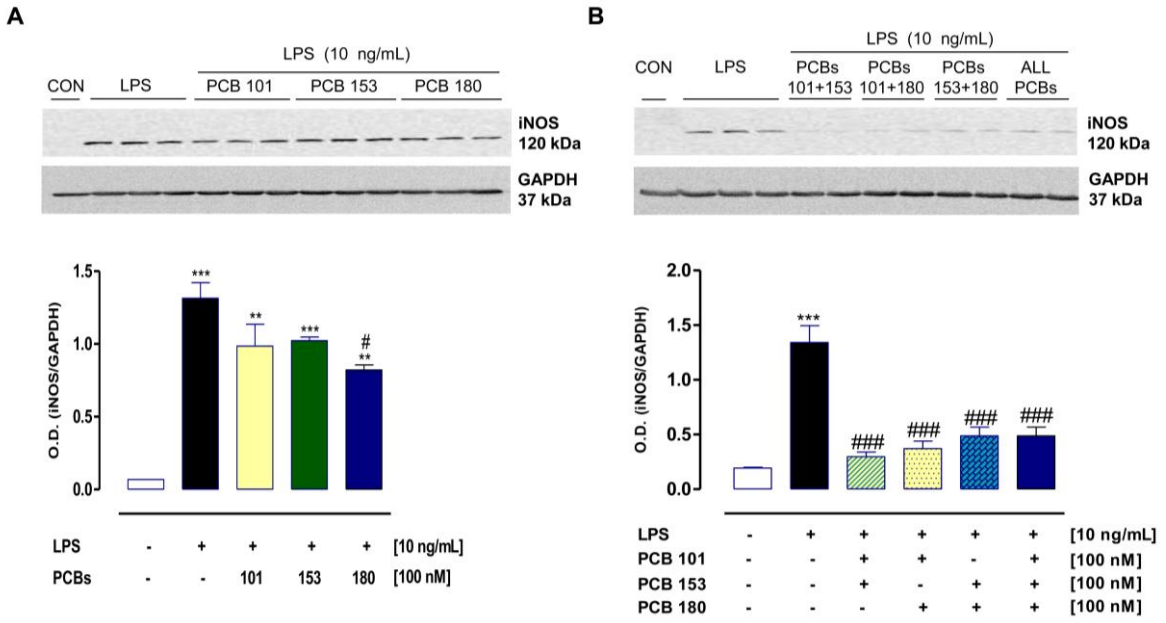


Fig. 26 Effects of NDL-PCBs 101, 153 and 180, alone (Panel A) or associated (Panel B), on LPS-induced iNOS protein expression in J774A.1 cells. Lysates were obtained from control and 24 h-treated cells with NDL-PCBs examined at 100 nM, alone or differentely combinated, in presence of LPS (10 ng/mL) stimuli. A representative immunoblot is shown. Densitometric analysis of protein bands was performed on three separate experiments. GAPDH protein immunoblot was performed to ensure equal sample loading. **P < 0.01, ***P < 0.001, vs control cells; $^{\#}$P < 0.05, and $^{\#\#\#}$P < 0.001 vs LPS treated cells.

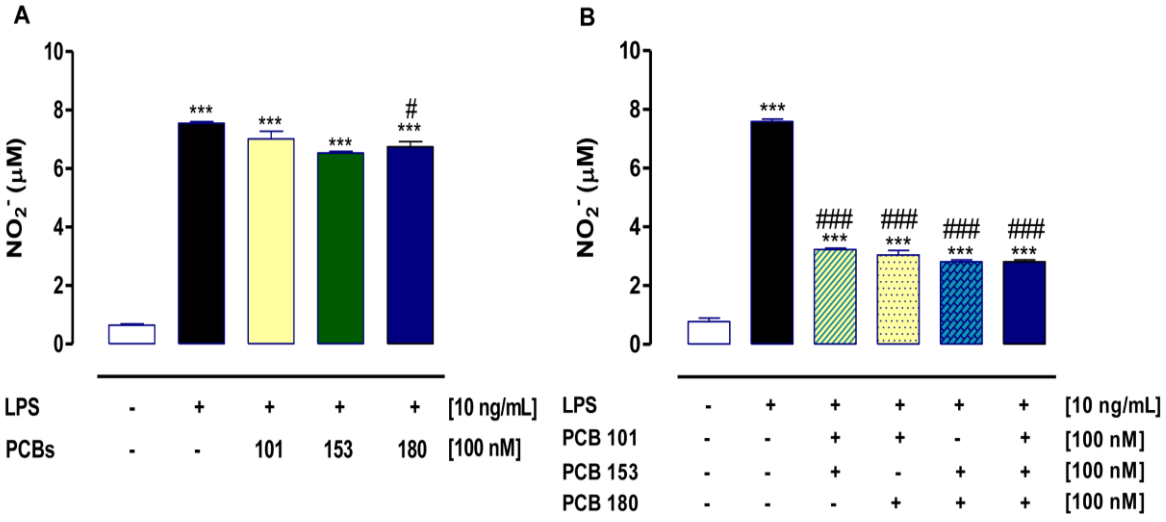


Fig. 27 Effects of NDL-PCBs 101, 153 and 180, alone (Panel A) or in association (Panel B) on LPS-induced NO^{2-} production by J774A.1. Cells were stimulated for 24 h with the aforesaid pollutants at 100 nM, both alone and in combination, in presence of 10 ng/mL LPS. The values are means of 3 determinations ± S.E.M. ***P < 0.001, vs control cells; $^{\#}$P < 0.05 and $^{\#\#\#}$P < 0.001 vs LPS treated cells.

7.2.5 Effects of NDL-PCBs 101, 153 and 180, alone or in association, on LPS-induced IkB-α degradation and nuclear p65 NF-kB expression

In order to assess the mechanism of action mediating immune suppression caused by NDL-PCB exposure in this macrophage cell line, we also investigated their ability to modulate the LPS-induced IkB-α degradation and nuclear p65 NF-kB content. As previously described these two proteins are involved in TLR-4 Mydd88-dependent and independent pathways and, interestingly, the p65 NF-kB traslocation into the nuclei, following pro-inflammatory stimuli, such as the LPS, leads to transcription of several innate immune response mediators.

IkB-α, physiologically, stays in cytoplasm binding to p65 subunit of p50 p65 NF-kB complexes, and inhibiting their translocation into the nuclei. Therefore IkB-α degradation is necessary to allow the NF-kB binding to its DNA responsive sequences. As expected, LPS induced a time-dependent and strong IkB-α degradation and, accordingly the bacterial endotoxin determined a contrary increase in p65 NF-kB expression in nuclear lysates (Figs. 28A and B). Its expression is significantly higher than the control cells after 30 minute LPS-stimulation ($P<0.001$). As shown in Figs. 23C-F, NDL-PCBs alone did not modify significantly the LPS-induced IkB-α degradation and NF-kB expression, while all the association reverted the effects of LPS on the expression of these two proteins.

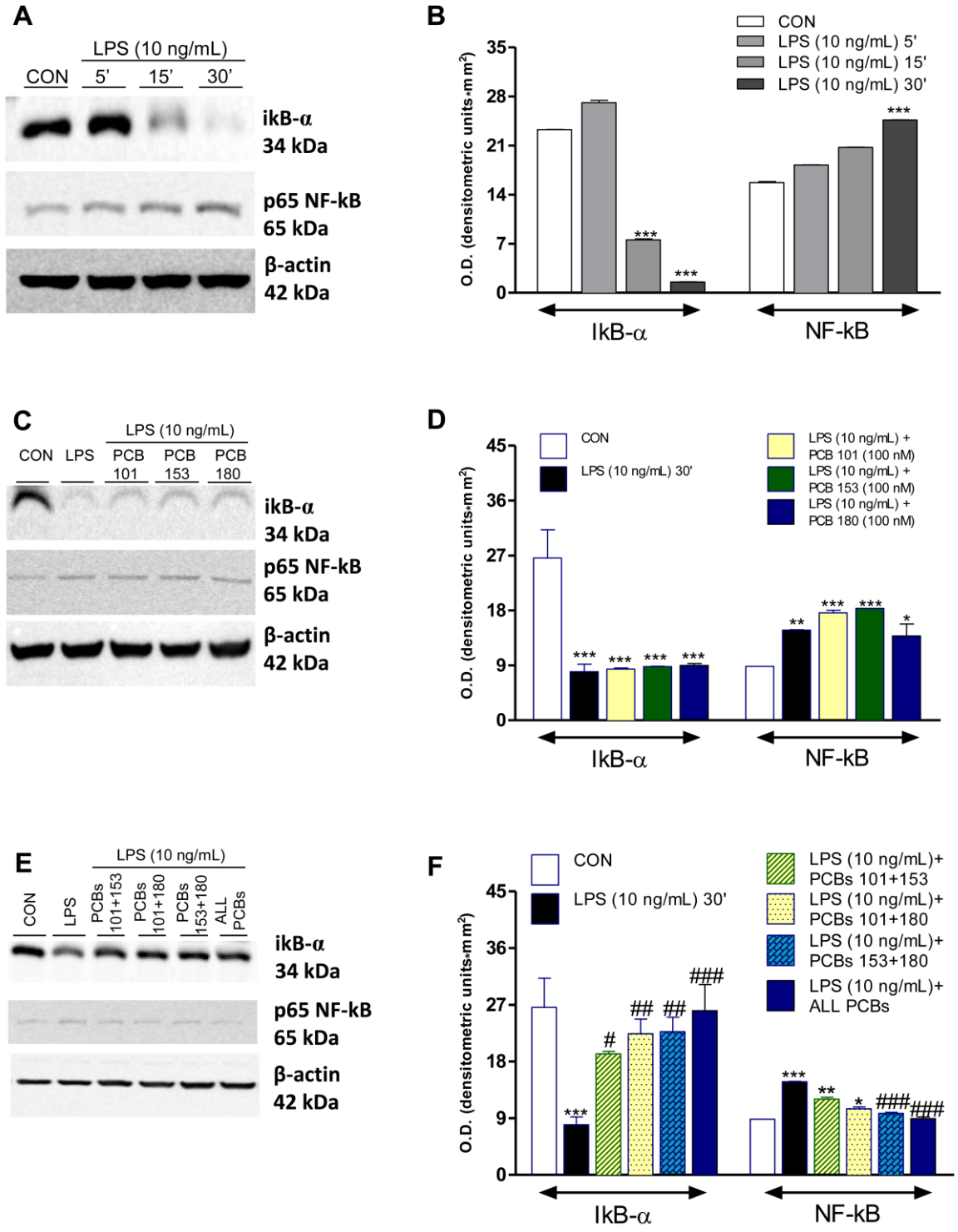


Fig. 28 Effects of NDL-PCBs, alone (Panels C and D) or in association (Panels E and F), on LPS-induced IkB-α degradation and nuclear p65 Nf-kB expression (Panels A and B). Lysates of cells were obtained from LPS-treated (10 ng/mL) macrophages for 5-15-30 min (Panels A and B) and from control or NDL-PCBs (100 nM) and LPS (10 ng/mL) costimulated macrophages for 30 min (Panels C-F). Representative immunoblots are shown. Densitometric analysis of protein bands was performed on three separate experiments. β-Actin protein immunoblot was performed to ensure equal sample loading. *P < 0.05, **P < 0.01, and ***P < 0.001 vs control cells; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 vs LPS treated cells.

7.3 Effects of NDL-PCBs 101, PCB 153 and PCB 180, alone or mixed, on mature adipocytes 3T3-L1: induction of leptin resistance

7.3.1 Effect of PCBs on adipocyte viability and lipid content

In these experiments all PCBs tested at all concentrations used (nM and µM range) did not modify cell viability (Figs. 29A and 29B). To examine the effects of PCB combination on cell viability, we incubated cells with the non toxic concentration (10-100 nM or 1 µM). Also when PCB 101, PCB 153 and 180 were differently combined, cell viability was not significantly reduced (Figs. 29C and 29D).

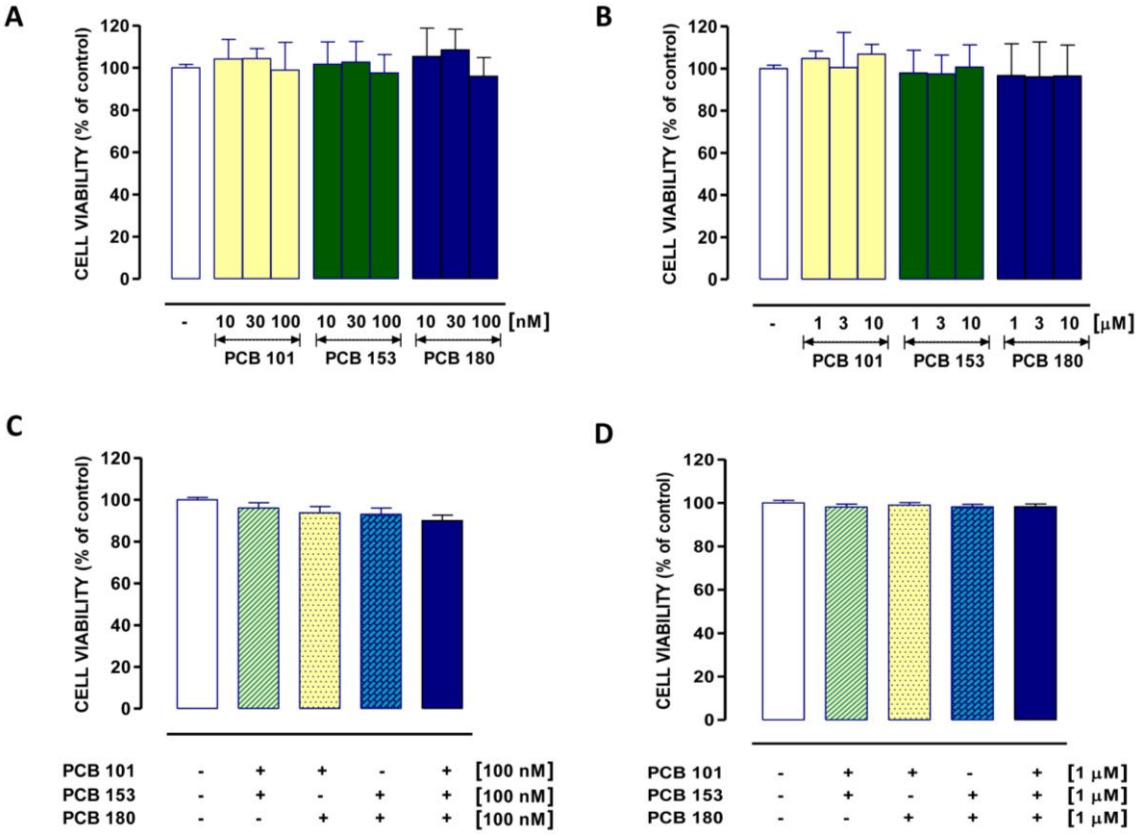


Fig. 29 Effects of PCB 101, 153 and 180 alone or in combination on adipocyte viability. 3T3-L1 cells were treated with PCBs at nanomolar (A) or micromolar (B) concentrations or in combinations two by two or all mixed 100 nM (C) or 1 µM (D) for 48h. Cell viability was evaluated using the MTT assay, as described in methods. The optical densities (OD_{620}) of PCBs treated cells were compared with the OD of the control wells, where cells received the vehicle. Each percent value is the mean ± SEM of three independent experiments, each performed using triplicate wells.

Then, we examined the effects of all PCBs (1μM) on differentiated 3T3-L1 adipocytes, evaluating the capability to accumulate fat, through ORO staining and PPARγ expression. A significant increase in lipid content was shown when cells were treated with PCBs alone (Fig. 30A) or in combination (Fig. 30B). Interestingly, the expression of PPARγ, a master regulator of adipocyte differentiation, was not significantly modified in all cell treatments (Figs. 30C and 30D).

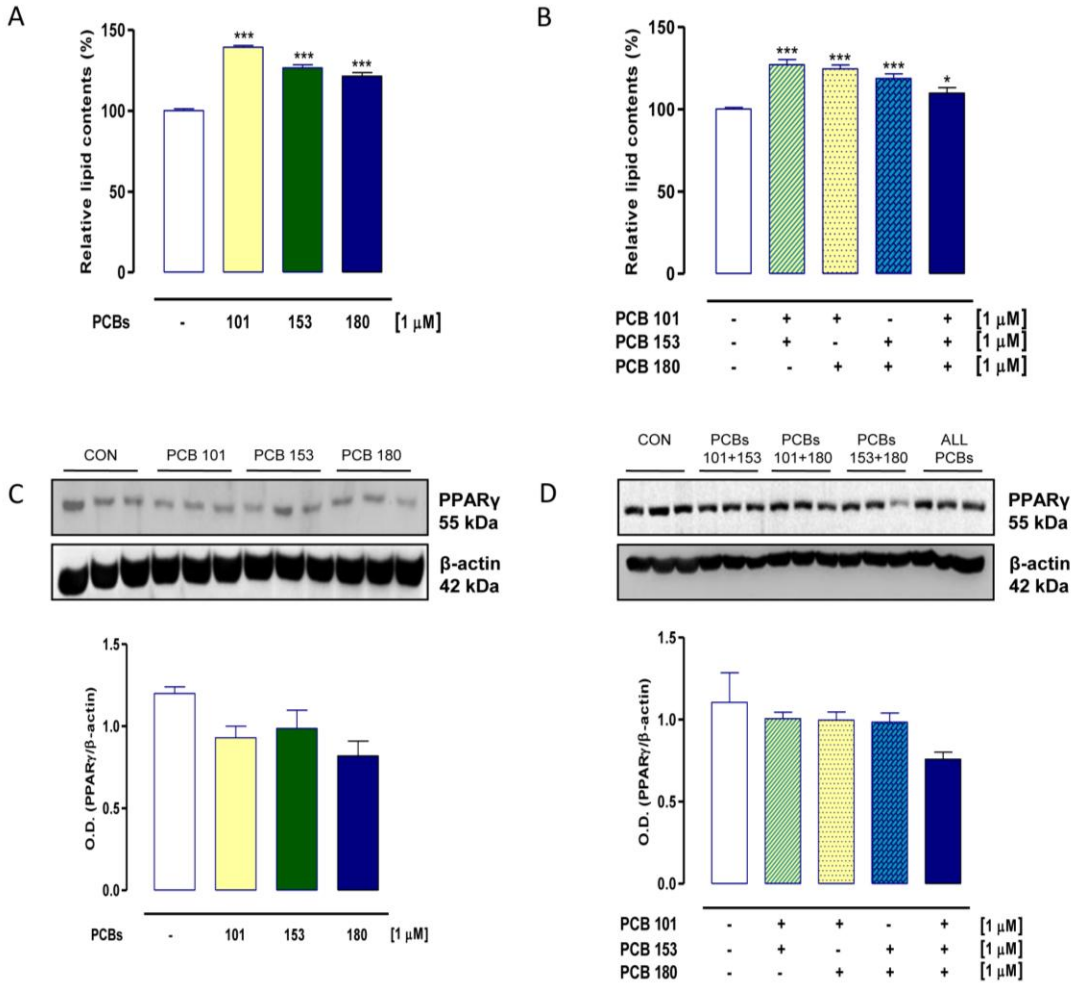


Fig. 30 Effects of PCBs alone or in combination on lipid content and PPARγ expression in mature adipocyte 3T3-L1. Cells, induced to differentiate for 7 days, were treated with PCBs alone (A) or in combination (B) for two days (1 μM). After PCBs treatment, cells were stained with Oil Red O. The optical densities (OD_{510}) of PCB treated cells were compared with the OD of the control wells. Results are expressed as mean ± SEM of three independent experiments, each performed using triplicate wells. Expression and densitometric analysis of PPARγ on cells treated with 1 μM PCBs alone (C) or in combination (D) are shown. β-actin expression was used as loading control. Shown is immunoblot representative of at least three separate experiments. *P< 0.05 and ***P<0.001 vs untreated cells.

7.3.2 Effect of PCBs on adipocyte leptin and its functional receptor ObRb expression

As shown in Fig. 31A, PCB 153, but not PCB 101 or PCB 180, significantly increased leptin transcription. Interestingly, the ability to promote leptin expression was significant when singly inactive PCB 180 and PCB 101 were combined, or associated to PCB 153 or all together (Fig. 31A). Conversely, PCBs alone did not modify the transcription of ObRb (Fig. 31B), while all PCB combinations significantly decreased the receptor expression (Fig. 31B).

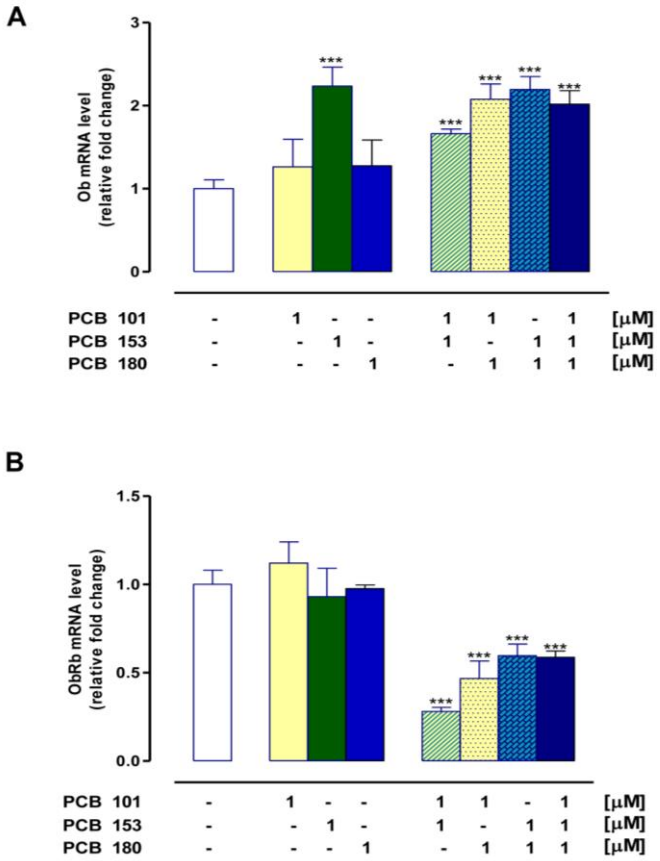


Fig. 31 Effects of NDL-PCBs on mRNA expressions of leptin (Ob) and its receptor (ObRb) in mature adipocytes. 3T3L1 differentiated adipocytes were exposed to NDL-PCBs at 1 µM, both alone and mixed, for 48 hours. After that, cells were lysed for collection of RNA performed as described in Materials and Methods section. The mRNA expression levels, expressed as relative fold change, of Ob (A) and its functional receptor ObRb (B) were analyzed by real-time PCR. Values are representative of at least three separate experiments. ***P<0.001 vs untreated cells.

7.3.3 Effects of PCBs on leptin signalling

To evaluate the induction of leptin resistance by combined PCBs, we studied ObRb signalling transduction, determining the phosphorylation of STAT3, a downstream activator of the transcription of leptin gene targets, and the expressions of PTP1B and SOCS3, two well-known inhibitors of JAK-STAT signalling cascade. The combination of PCB 153 and 180 or all PCBs together induced a significant decrease in the activation of STAT3, evidenced by a reduction of the phosphorylated protein (Fig. 32A) paralleled by an increase of PTP1B expression (Fig. 32B) and SOCS3 (Fig. 32C).

To test the direct effect of the PCB 153 and 180 association and all mixed congeners on leptin signaling pathway, we examined the impact of these combinations on STAT3 phosphorylation in response to leptin stimulation in mature adipocytes. As shown in Figure 32C, leptin mediated STAT3 phosphorylation in time-dependent manner and the association examined significantly inhibited leptin-induced STAT3 activation after 10 hour exposure ($P<0.001$).

7.3.4 Modulation of AMPK activation and ACC activation by PCBs

Since leptin effect on adipose tissue is orchestrated by activation of AMPK/ACC pathway we evaluated the phosphorylated status of both enzymes. The evaluation of AMPKα activation revealed a marked reduction in the phosphorylated protein in PCB treated adipocytes (Fig. 33A), that was associated to a decrease in ACC activity, in particular, when PCB 153 and PCB 180 were combined or all mixed (Fig. 33B), suggesting a decrease in lipid catabolism.

7.3.5 Effects of PCBs on cytokine expression

As well known IL-6 and TNF-α, two pro-inflammatory cytokines produced by adipose tissue, play a key role in the onset of metabolic dysfunction related to type 2 diabetes or metabolic syndrome. PCBs exposure induced both alone and in combination the increase in IL-6 (Fig. 34A) and TNF-α (Fig. 34B) levels. The increase in IL-6 mRNA expression was highly significant only for PCB 153 and PCB 180 alone and all mixed.

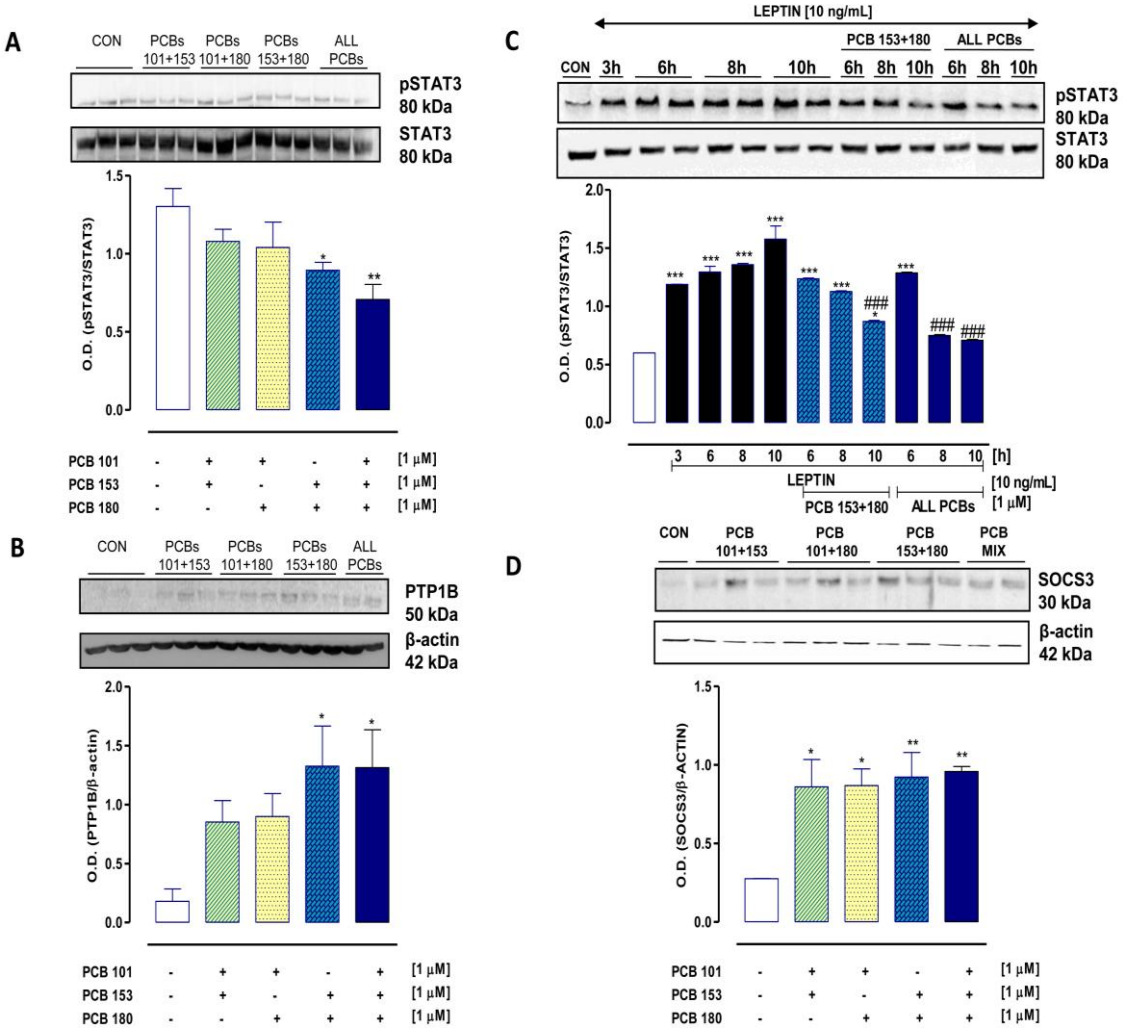


Fig. 32 Effects of PCBs on leptin signalling in mature adipocytes. Levels of pSTAT3 (A), PTP1B (B) and SOCS3 (D) in cells treated with PCBs in combination (1 μM) for two days are shown. Temporal expression of pSTAT3 in leptin (10 ng/mL) and PCBs (153+180 and all mixed) co-treated mature adipocytes is also shown in Panel C. Representative blots of pSTAT3 and pSTAT3/STAT3 ratio from densitometric analysis of bands of all samples are shown. Immunoblots representative of at least three separate experiments are shown. β-actin protein expression was used as loading control. *P< 0.05 vs untreated cells.

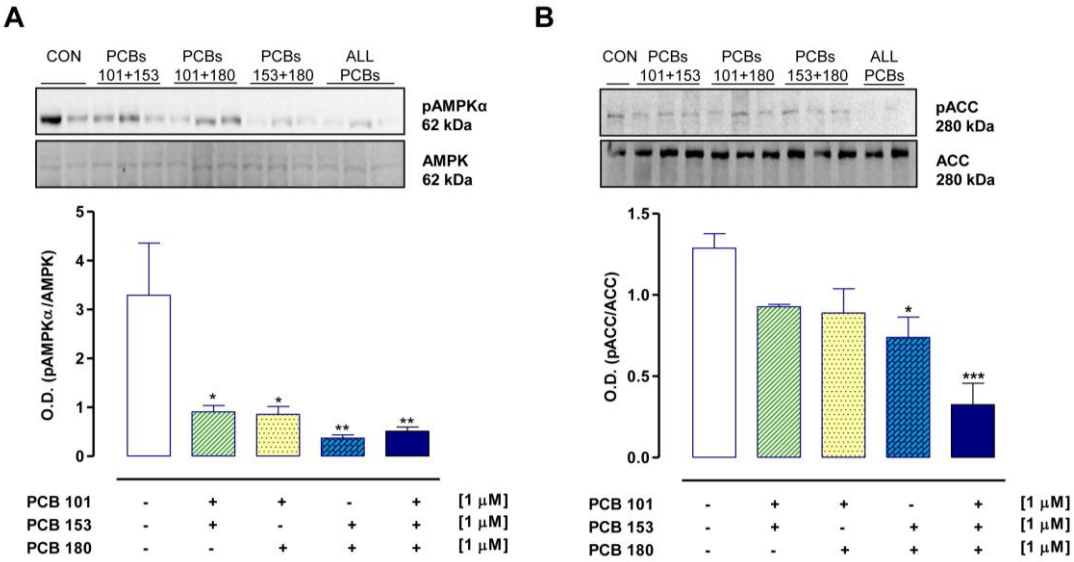


Fig. 33 Effects of PCBs on AMPK-ACC pathway. Changes in pAMPKα (A) and pACC (B) in mature adipocytes treated with PCB 101, 153, and 180 in combination (1 μM) for two days. Depicted are a typical display of phosphorylated protein expression and pAMPKα/AMPK and pACC/ACC ratio from densitometric analysis of bands of all samples. Data are given as mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001 vs untreated cells.

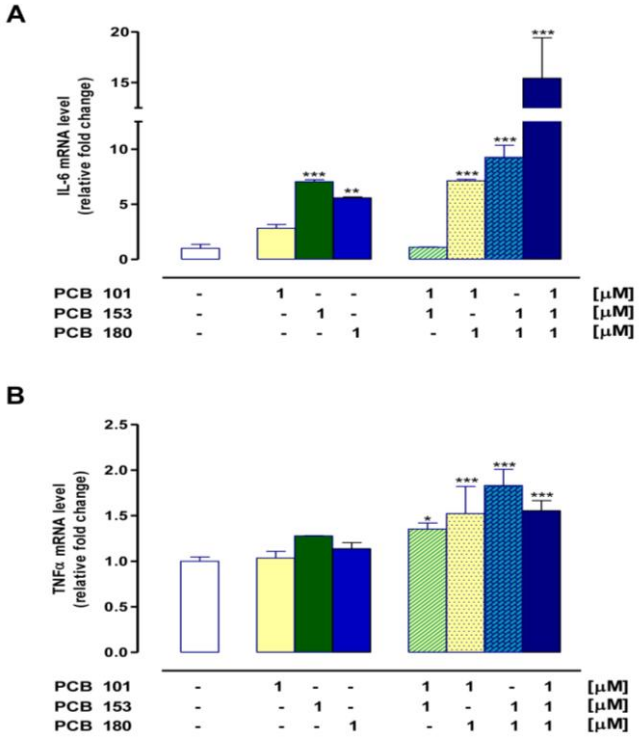


FIG. 34 Effects of NDL-PCBs on mRNA expressions of interleukin (IL)-6 and tumor necrosis factor (TNF)α in mature adipocytes. 3T3L1 differentiated adipocytes were exposed to NDL-PCBs at 1 μM, both alone and mixed, for 48 hours. After that, cells were lysed for collection of RNA performed as described in Materials and Methods section. The mRNA expression levels, expressed as relative fold change, of IL-6 (A) and TNFα (B) were analyzed by real-time PCR. Values are representative of at least three separate experiments. *P<0.05, **P<0.01, and ***P<0.001 vs untreated cells.

7.4 NDL-PCBs 101, 153 and 180 induce apoptosis of chondrocytes via oxidative stress

7.4.1 Effects of PCBs on cell viability

As shown in Fig. 35 (A-B), treatment of murine ATDC5 and human T/C-28a2 chondrocytes with increasing concentrations of NDL-PCBs 101, 153 and 180 (1, 5 and 10 µM) for 24 hours induced a significant decrease of cell viability, determined by MTT assay. In a similar way, as shown in Fig. 35 (C-D), cell viability reduction was more evident after 48 h of exposure to pollutants.

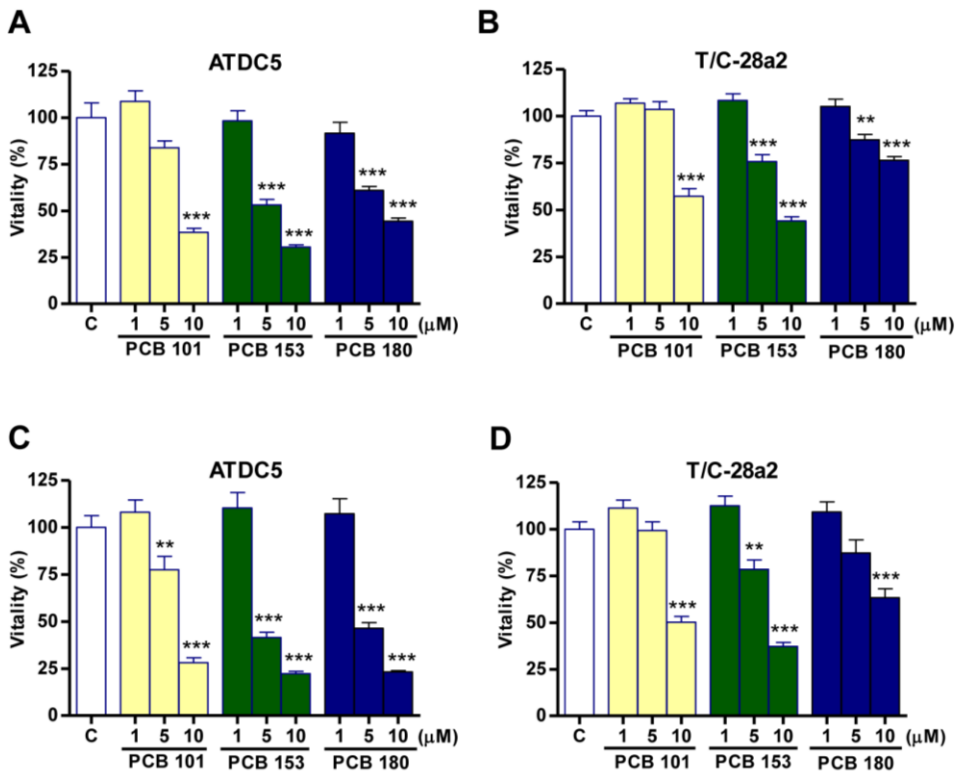


Fig. 35 Effects of PCB 101, 153 and 180 alone on cell viability. Cells incubated with PCBs (1-5-10 µM) for 24 h (A-B) and 48 h (C-D) were analyzed using a MTT colorimetric assay. Results represent mean ± SEM of at least 3 independent experiments, each with at least 8 independent observations. Asterisks indicate significant differences from control with DMSO 0.1% (**P < 0.01, ***P < 0.001).

7.4.2 Effects of PCBs on LDH levels

To evaluate whether the cytotoxic effect of NDL-PCBs analyzed was associated with necrosis, we performed the lactate dehydrogenase (LDH) assay. As shown in Fig. 36, in line with the above showed results on cell viability, LDH release was significantly increased after 24 h exposure to all NDL-PCBs tested so far both in ATDC5 cells (Fig. 36A) and human T/C-28a2 chondrocytes (Fig. 36B). Interestingly, our results indicated that the LDH release increased for all PCBs in a concentration dependent manner.

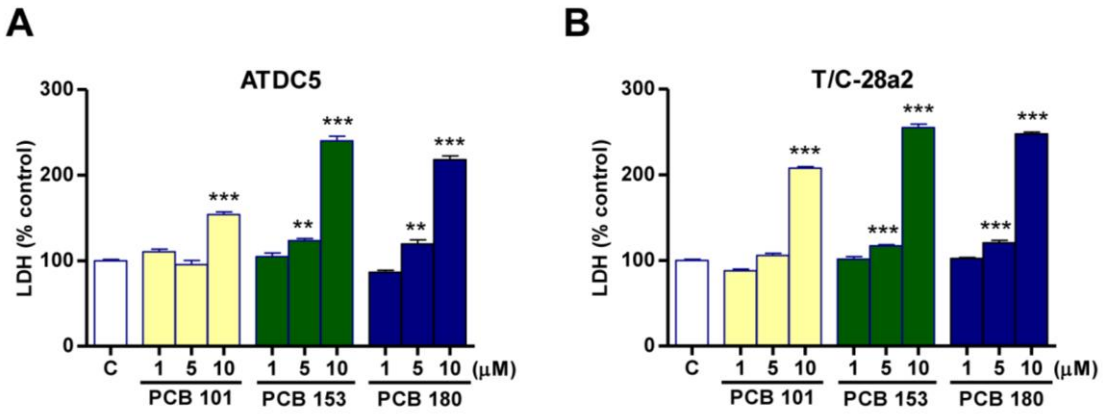


Fig. 36 Effects of PCB 101, 153 and 180 on LDH levels. Undifferentiated ATDC5 cells and human T/C-28a2 chondrocytes were incubated in absence (DMSO 0.1%) or presence of PCBs (1-5-10 µM) for 24h. Then, supernatants were collected and analysed by LDH assay. Results are expressed as means ± SEM of 3 independent experiments. ** P<0.01, *** P<0.001 vs control with vehicle (DMSO 0.1%).

7.4.3 PCBs induce necrosis and late apoptosis in ATDC5 cells

To gain further insights into the mechanism of cell death elicited by PCBs on ATDC5 cells and to determine whether NDL-PCBs may also induce also apoptosis, Annexin V-FITC/propidium iodide (PI) staining of ATDC5 cells was performed. As shown in Fig. 37, all PCBs tested so far showed an increase in the percentage of PI-positive/Annexin-V-negative cells at 10 µM (Quadrant 1). Intriguingly, a slight increase in Annexin-V-positive/PI-positive cells (Quadrant 2) suggested a potential involvement of

late apoptosis mechanism. However, this effect is not statistical significant and needs further demonstrations.

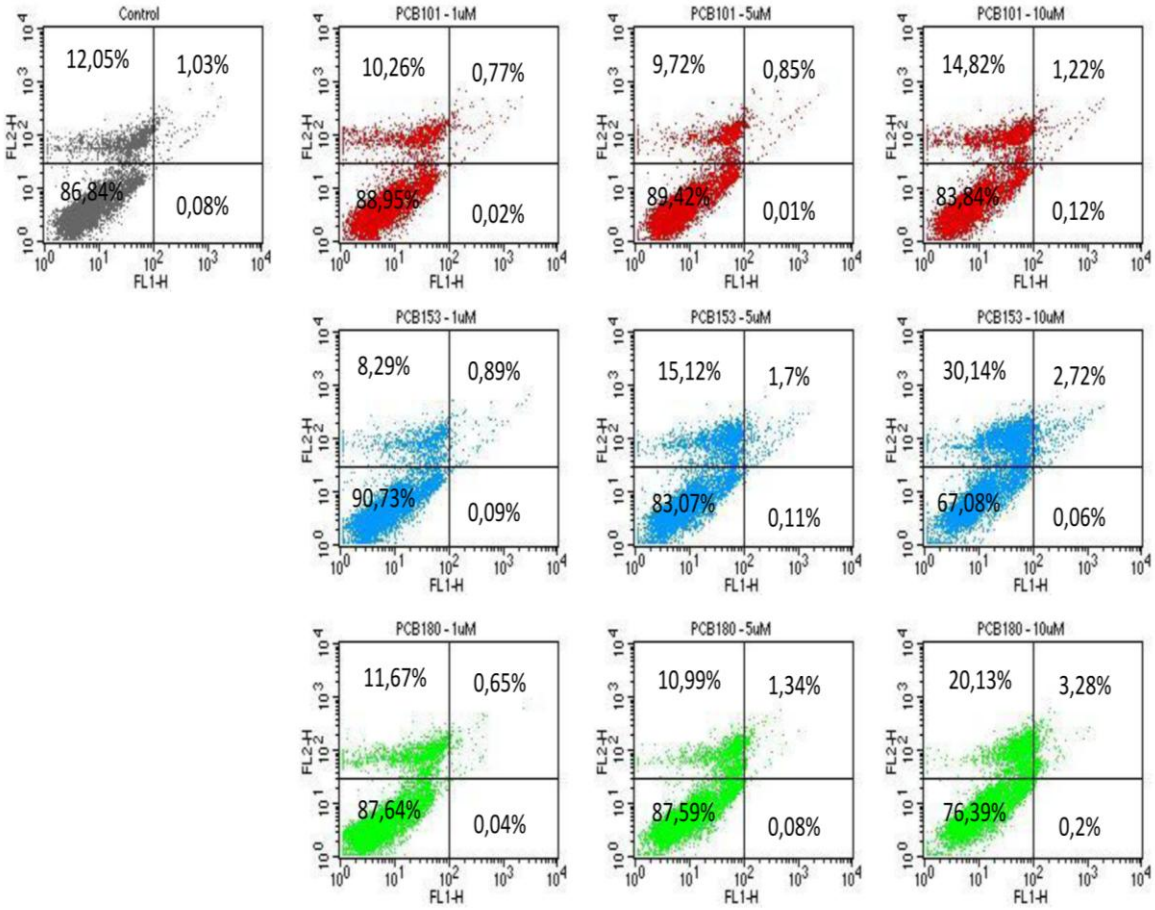


Fig. 37 In vitro assessment of apoptosis in undifferentiated ATDC5 cells incubated with PCBs at increasing concentrations (1, 5 and 10 µM) for 24h. The induction of apoptosis was determined by flow cytometric analysis of Annexin V-FITC and PI-staining. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI-positive, late apoptotic cells. Finally, the cells in the upper left quadrant indicate PI-positive, necrotic cells.

7.4.4 Caspase-3 activation by PCBs in ATDC5 cells

Since caspase-3 activation by proteolytic cleavage is considered a break point in apoptosis induction, we also assessed the effect of PCBs (1-5-10 µM) on caspase-3 cleavage by Western blot analysis. As shown in Fig. 38, all PCBs tested so far significantly increased the expression of cleaved caspase 3 in a concentration-dependent manner.

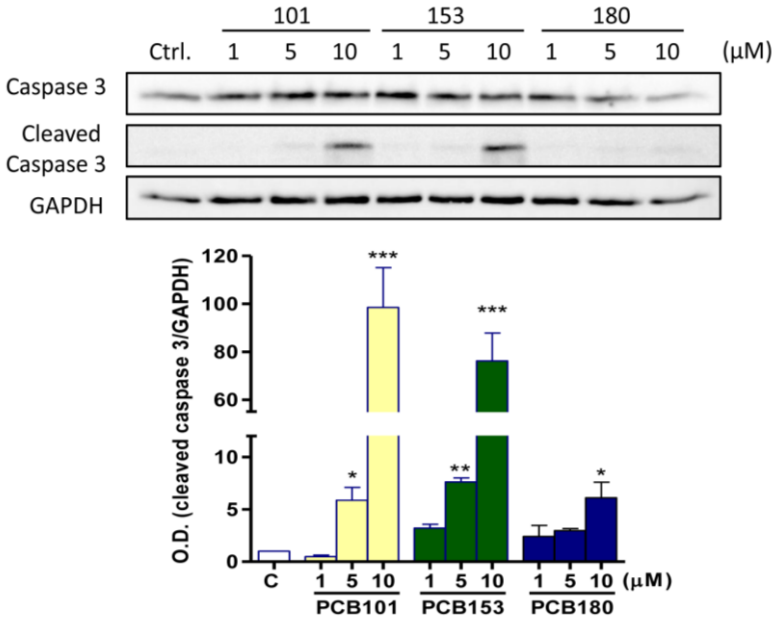


Fig. 38 Induction of caspase 3 cleavage in ATDC5 cells by PCB 101, 153 and 180. Cells were incubated with PCBs for 3 h. Cell lysates underwent Western blotting analysis using cleaved and total caspase 3. GAPDH antibody was used as loading controls. Blots are representative of at least three independent experiments. Western blot densitometric analysis is also reported (n=3; differences vs control, *P <0.05, **P < 0.01, ***P < 0.001).

7.4.5 PCBs affect Bcl-2 and Bax expression in ATDC5 cells

In order to clarify further the PCB-driven apoptotic pathway, we analyzed also the intrinsic pathway activation by considering Bax and Bcl-2 proteins expression.

As shown in Fig. 39, after 3 h of PCB exposure, we observed an altered expression of Bcl-2 and Bax. To note, the Bcl-2/Bax ratio decreases in a concentration-dependent manner, suggesting that the intrinsic pathway of PCB-driven apoptosis is also in play.

7.4.6 PCBs induce p38 phosphorylation in ATDC5 cells

p38 MAPK has been associated with the induction of apoptosis (Thornton and Rincon, 2009). Thus, we examined p38 activation in PCB-treated ATDC5 cells. As shown

in Fig. 40, all PCBs tested so far increased p38 phosphorylation in a concentration-dependent manner. This effect was statistically significant at 10 µM.

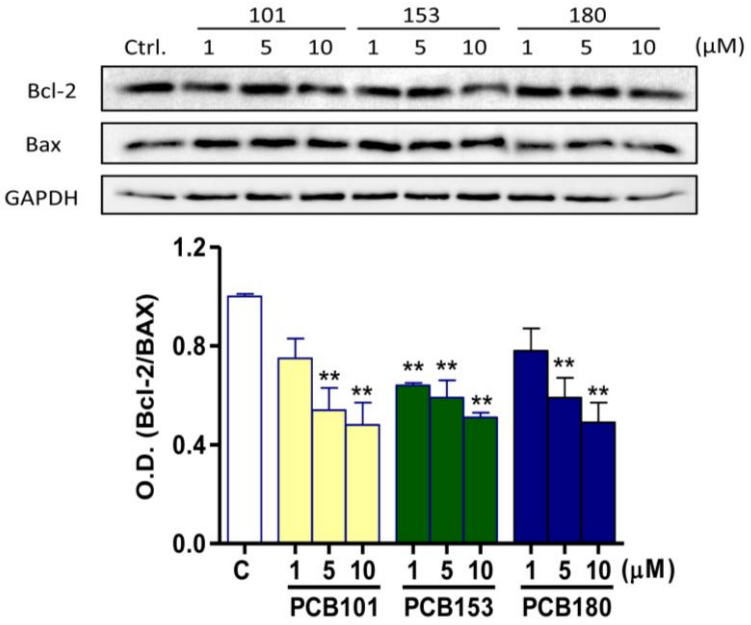


Fig. 39 Induction of apoptosis signalling molecules in ATDC5 cells by PCB 101, 153 and 180. Cells were incubated with PCBs for 3 h. Cell lysates underwent Western blotting analysis using Bcl-2 and BAX. GAPDH antibody was used as loading controls. Blots are representative of at least three independent experiments. Western blot densitometric analysis (n=3; differences vs control, **P < 0.01).

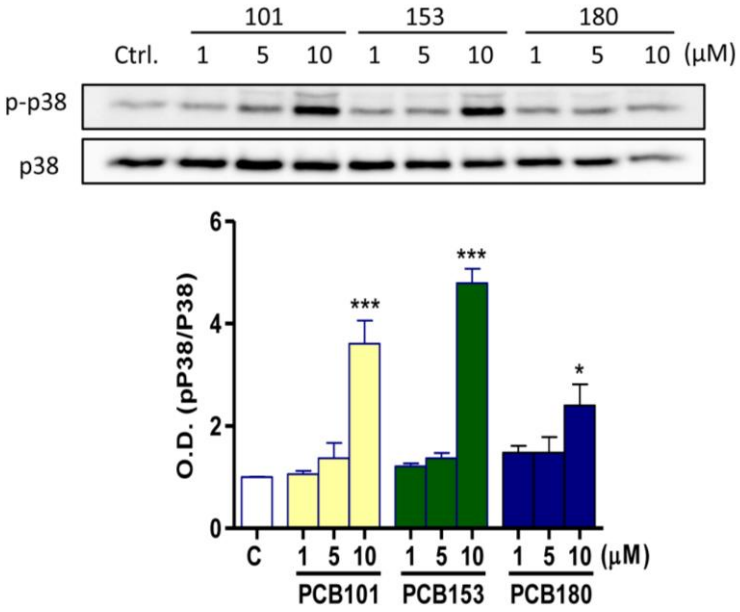


Fig. 40 Induction of apoptosis signalling molecules in ATDC-5 cells by PCB 101, 153 and 180. Cells were incubated with PCBs for 3 h. Cell lysates underwent Western blotting analysis using phospho-P38 antibody. P38 antibody was used as loading controls. Blots are representative of at least three independent experiments. Western blot densitometric analysis (n=3; differences vs control, *P <0.05 and ***P < 0.001).

7.4.7 NDL-PCBs induce apoptosis via oxidative stress in ATDC5 cells

To determine whether mechanisms of cell death driven by PCBs were also associated with the oxidative stress induction, we evaluated malondialdehyde (MDA) content in ATDC5 cell lysates.

As shown in Fig. 41A, all NDL-PCBs tested so far induced a concentration-dependent increase of MDA levels, being statistically significant at 10 μM.

Furthermore, in order to establish whether PCB-induced MDA increase was associated with an imbalance of the antioxidant system, we also evaluated the Total Antioxidant Status (TAS). As shown in Fig. 41B, a strong and dose-dependent reduction in TAS levels was observed in ATDC5 cells after NDL-PCB challenge. Conversely, but in agreement with above mentioned results, the Ossidative Stress Index (OSI; Fig. 41C) was increased after PCB treatment in a concentration dependent manner.

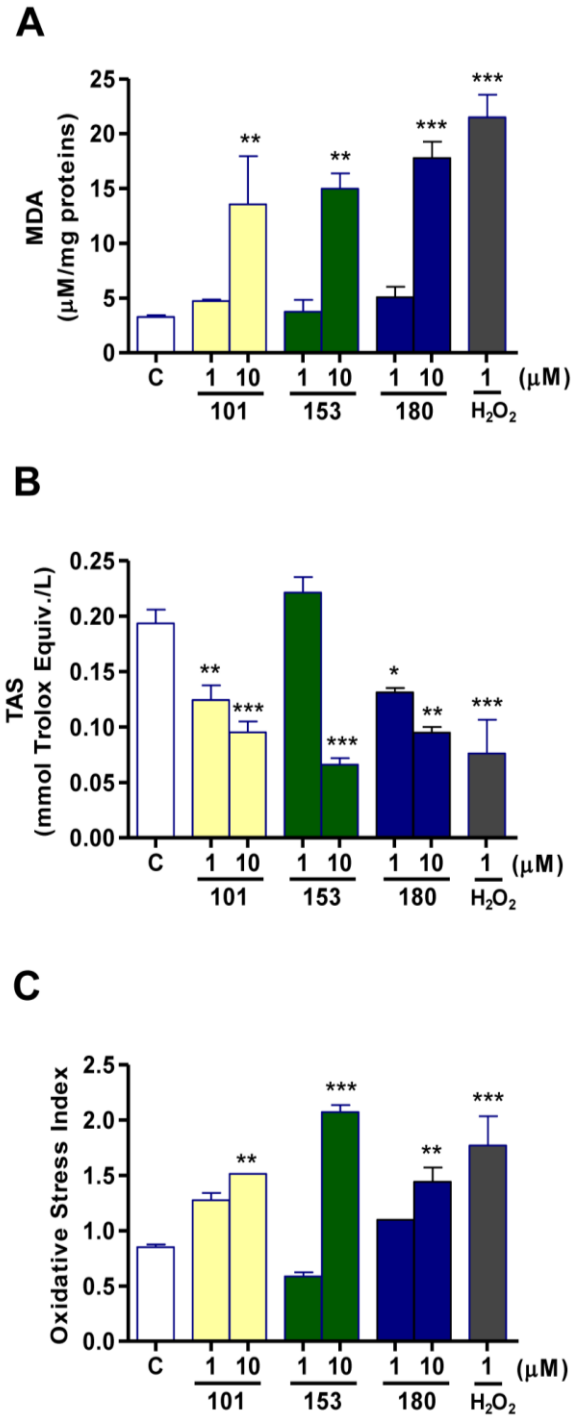


Fig. 41 Evaluation of oxidative stress in ATDC-5 cells exposed to PCB 101, 153 and 180. Cells were incubated with the aforesaid NDL-PCBs for 24h (1-10 µM). H_2O_2 treated cells were used positive controls. Afterwards, malondialdehyde (MDA) content was assessed as described in Methods Section (Panel A), as well as Total Antioxidant Status (TAS, Panel B) and Oxidative Stress Index (Panel C). Results represent mean ± SEM of at least 3 independent experiments, each with at least 3 independent observations. ** P<0.01, *** P<0.001 vs control (Panel A and B), * P<0.05, ** P<0.01, *** P<0.001 vs vehicle (DMSO 0.1%) control cells (Panel C).

8. DISCUSSION

Few studies have been published on the levels of OCPs and PCBs in fish species in Campania region. The presence of OCPs and PCBs in the edible tissues of two bivalves species from the coastal area of Castelvolturno in Gulf of Naples suggest that such compounds likely contribute to the heavy pollution of the Campania aquatic ecosystem causing a possible health hazard. Thus, we argue that the monitoring activity is a matter of importance.

The predominance of hexa-chlorinated PCBs in mussels, especially PCB 153 and PCB 138, has been showed for other coastal locations along the western Mediterranean (Castro-Jimenez et al., 2008; Villeneuve et al., 1999) and the Adriatic Sea (Bayarri et al., 2001; Piersanti et al., 2006). This predominance is due to the presence of chlorines at positions 2, 3 or 5 in one or both rings that makes the congener refractory to metabolic attack by monooxygenases and slow to be eliminated (Walker, 2001).

It is rather difficult to interpret the differences in residue levels observed in bivalves regarding PCBs as well as POCs. The habitat may provide a first explanation for the different OCs patterns detected in the two species analysed.

The saltwater mussel *Mytilus galloprovincialis* thrives in an intertidal habitat characterized by a dynamic environment with changing temperature and salinity, turbidity, and regular episodes of exposure to surrounding air owing to tides. Because mussels filter large amounts of water for both feeding and respiration, they are directly exposed to contaminants from both dissolved or particulate (mainly finest sediments) phase in the water column. The benthic mollusc *Ensis siliqua* filters water and is exposed to the organic

matter, living in strict contact with the coastal medium and fine size sediments (Dame, 1996).

Different patterns of PCBs congeners between the two species may be due to the preferential bound of the medium chlorinated congeners (e.g. PCB 138, 153, 180) to the smaller fractions of the sediment (Piérard et al., 1996). It is likely that tidal movements and other disturbances of the sediment floor tend to resuspend fine size sediments, thus making the above mentioned PCBs more available to mussels than clams. In fact, in the present study the PCB 180 was significantly higher in mussels than clams, the values being 3% and 1%, relatively to ∑PCBs, respectively. Moreover, our evidence accords with that by Thompson et al. (1999), who observed higher levels of PCBs in farmed mussels respect to the sediment dwellers species razor and carpet shells and, in particular, the dominant presence of PCB 153 followed by PCB 138 in mussels and the prevalence of PCB 118 followed by PCB 138 and PCB 153 in the benthic species.

The significantly lower presence of lighter congeners in mussels might be due to the partial volatilization of the not-bounded fraction of these compounds caused by weathering and other more specific factors, such as aquatic movements occurring during shipping activity (Ailstock et al., 2002). Moreover, according to Piérard et al. (1996) these congeners are easily linked to the coarsest sediments less susceptible to resuspension, thus being less bioavailable for the water column bivalves.

Regarding the difference in octa-chlorobiphenyls levels, the congeners — characterized by high octanol-water partition coefficient levels, from 7.62 to 7.80 — are usually absorbed by sediments, thus only a small fraction remains in the dissolved form. These congeners don’t appear to be preferentially linked to the smallest fractions of the

sediments (Piérard et al., 1996) and they probably settle near their source (Hong et al., 2003).

One explanation, on the other hand, for the relatively high OCPs levels in clams is that the sampling was performed along the coast, in proximity of the outlet of canals draining irrigation waters of neighbouring farmed fields. Thus, the pesticides are poured in the sea entering directly in contact with the species living in the sandy bottom near the shore. Further, the higher presence of Dieldrin and p,p'-DDT in mussels as well as the prevalence of the former, among all pesticides analysed, may be explained by the different behaviour of the OCPs in the aquatic environment. To describe the distribution of the compounds between particulate and aqueous phases in the equilibrium is used the partitioning factor K_d. Carvalho et al. (2009) used this parameter to explain the differences observed in the OCPs bioaccumulation by oysters and fish. In particular, as the K_d values for Dieldrin and p,p'-DDT were lower than those for p,p'-DDD and p,p'-DDE, they concluded for an higher presence of the first (second) two pesticides in the aqueous (sediment) phase. This is consistent with our evidence of more supply of Dieldrin and p,p'-DDT in mussels and of p,p'-DDD and p,p'-DDE in the clams.

Recently, it has been stressed that the patterns and levels of hydrophobic pollutants in aquatic organisms are determined not only by their concentrations in external environmental compartments but also by physiological processes, such as biotrasformations. There are metabolic differences among species that may result from either differential uptake mechanisms or different titres of relevant enzyme systems in any organism (Katagi, 2010).

The bivalves show a very limited capacity to metabolise PCBs through the cytochrome P450 system (Sheehan et al., 1995); hence, the concentrations of any PCB

congener, as well as the total congeners pattern, are scarcely modified by biotrasformations. About the differences in the pesticides levels, an explanation might be related to the species-specific variability in glutathione-*S*-transferases (GST), the enzyme that convert both p,p'-DDT and p,p'-DDE into water-soluble metabolites. Some researchers observed a modification in the GST levels in bivalves exposed to OCPs as the consequence of an adaptive response to pollutants in the immediate environment (Sheehan et al., 1995), suggesting the possible use of this parameter as a biomarker of the exposition (De Luca-Abbott et al., 2005). There are few studies, however, about the enzymatic activities regarding the OCs metabolism in the clams as well as experiments comparing the enzymatic levels of *Mytilus galloprovincialis* and *Ensis siliqua*, to explain the different residue levels revealed as a reduced GST presence in clams.

Dealing with two bioindicator species coming from an unexplored coastal area of the Campania region, we showed relevant residue levels of OCs in their soft tissues. Observed differences might be mainly related to the peculiar PCBs and OCPs sediment-water partitioning. In particular, the levels detected suggest that OCs are likely to contribute to the heavy pollution of the Campania aquatic ecosystem, even though a decreasing trend seems to be probable.

Conversely, observed PCBs residue concentrations may represent a human health hazard, since they exceed the recently limits established by EU. Our results also showed that the PCBs 118, 138, 153, and 180 were the most abundant congeners in the bivalves analyzed. Interestingly, these PCBs have been found at high concentrations in human adipose tissue from Italian surgical patients (Corsolini et al., 1995). Since PCBs as well as OCPs accumulate in organisms through the food chain, the diet, in particular animal fats and fish, is considered the main source of human exposure to PCBs for western countries

(Duarte-Davidson and Jones, 1994; Harrad and Smith, 1999; Zuccato et al., 1999; Liem et al., 2000; Bayarri et al., 2001; Bilau et al., 2007).

In this study, we not only evaluated the OC bioaccumulation, but we also investigated the effects of NDL-PCBs 101, 153 and 180, alone or differently associated, on immune and endocrine systems, since these pollutants are currently considered as EDCs by the scientific world (El-Shahawi et al., 2010).

As reported by Igarashi et al. (2006) EDCs may exacerbate infectious diseases because of their ability to disturb the immune system by interfering with endocrine balance. Infectious diseases are frequently caused by microbial invasion. To overcome this invasion, the human body utilizes a sophisticated immune response. Disturbance of this well-orchestrated immune response may results in the development of serious infectious diseases. In agreement, several epidemiological studies demonstrated a positive correlation between long-term PCB exposure and a weaking of immune defences and increased incidence of ear or respiratory infections (Weisglas-Kuperus et al., 2000, 2004; Dallaire et al., 2006; Dewailly et al., 2000), reduced functional capacity of lymphocytes, as indicated by decreased responses to mitogen stimulation (Belles-Isles et al., 2002) and insufficient antibody response to vaccination (Heilmann et al., 2006, 2010).

Though toxicological studies on NDL-PCBs are in their infancy, our recent *in vitro* data demonstrate the ability of NDL-PCBs to suppress the macrophage response and support an apoptotic mechanism initiated by PCBs 101, 153, and 180 (Ferrante et al., 2011). Additionally, Levin et al. (2005a) showed that exposure of healthy human leukocytes to the NDL-PCBs 138, 153, and 180 resulted in reduced phagocytosis. Conversely, no mechanistic and *in vitro* studies, assessing the influence of NDL-PCBs 101, 153 and 180 on the immune innate function of macrophages, are available.

As is well known, macrophages play an important role in the host's immune defense mechanisms, recognizing patterns on foreign biological substances through the activation of pattern-recognition receptors such as TLRs (Schwartz and Svistelnik, 2012). In particular, macrophages are activated by LPS, the major molecular component of the outer membrane of Gram-negative bacteria and a potent natural immune stimulator (Fujihara et al., 2003), which was identified as a ligand of TLR-4.

Normally, LPS/TLR-4 interaction initiates various signalling pathways leading to production of proinflammatory cytokines, such as IL-6 and TNF α, and chemokines, such as MCP-1 (Peri et al., 2012; Rodriguez-Vita and Lawrence, 2010). Their genes collectively ramp up the host's immune defense mechanisms. It is noteworthy that in our experimental condition the LPS-induced IL-6, TNFα and MCP-1 gene expression (reaching the statistically significance after 4 hour LPS-stimulation as well as Huang et al. (2012) has previously described was suppressed by NDL-PCBs, especially as mixtures, showing a strong synergistic effects. Indeed, the penta-chlorinated congener was the less immunotoxic compound besides PCB analyzed, whilst both PCB 153 and 180 significantly suppressed LPS-induced IL-6 expression. Moreover, the hexa-chlorinated congener reduced LPS-induced MCP-1 mRNA level and the heptachlorinated, on the other hand, suppressed TNF-α expression. Our results are in accordance with those by Hong et al. (2004), which showed that several EDCs were able to reduce or counteract the LPS-induced expression of TNFα in the mouse macrophage cell line RAW 264.

Immunocompetent cells, such as macrophages, have a wide repertoire of chemical signals to communicate with the other ones. For example, macrophages stimulated by LPS generate excess NO through the action of iNOS, phenotypic of classically activated macrophages (Zhou et al., 2014). NO have been involved in the earlier phases of innate

immune response to bacterial infection, playing a significant role in microorganism clearance. Indeed, here we showed the NDL-PCBs, especially in association, strongly inhibit LPS-induced NO^{-2} production. Our data are consistent with those by Hong et al. (2004) and Yoshitake et al. (2008), which highlighted the ability of several EDCs to suppress NO production in LPS-stimulated macrophages, suggesting that they interfere with NO-mediated signalling and host defense system against foreign pathogens.

Moreover, during inflammation, such as in the process of host defense, endotoxins and cytokines induce rapid alterations in cellular early gene expression, leading to the *de novo* synthesis of COX-2 (Emami et al., 2010) and iNOS (Meli et al., 2000). Co-induction of iNOS and COX-2 has been shown in several cell types, including murine macrophages (Ferrante et al., 2008) with similarities in the signal transduction pathways. Our data clarly demonstrated that J774A.1 macrophages exposed to NDL-PCB mixtures were less or not able to orchestrate a proper immune response against LPS, since also the bacterial endotoxin-induced COX-2 and iNOS expressions were suppressed by the simultaneous incubation of this cell line with pollutant associations and LPS.

Since the activation of the transcriptional factor NF-kB is essential for cytokine synthesis and nitric oxide production, in the present study we investigated the effects of NDL-PCBs on bacterial component-induced activation of NF-kB.

As expected, LPS induced a time-dependent and strong IkB-α degradation and, accordingly, in our experimental conditions, the bacterial endotoxin determined a contrary and significant increase in p65 NF-kB expression in nuclear lysates. Whilst the NDL-PCBs alone did not modify significantly the LPS-induced IkB-α degradation and NF-kB expression, all the association reverted the effects of LPS on the expression of these two proteins. Other researchers, in agreement with our data, demonstrated that several EDCs

possess the ability to inhibit bacterial component-induced activation of NF-kB, suggesting their potential to exacerbate infectious diseases (Igarashi et al., 2006; Ohnishi et al., 2008).

Taking together into account, our results showed for the first time that NDL-PCB mixtures reduced ability of macrophages to respond properly to noxious stimuli, such as LPS, by interfering with NF-kB activation. Thus, NDL-PCBs might play important roles in the host defense system against foreign pathogens, since they suppress the immune innate response, which physiologically is orchestrated against a xenobiotic antigen.

As NDL-PCBs 101, PCB 153 and PCB 180 are immunotoxic compounds and, because of their high lipophilicity, tend to accumulate in lipid-rich tissues of organisms that contaminate, we also evaluated the alteration of the mature 3T3-L1 adipocyte metabolism induced by these pollutants alone or associated two by two or all together.

To our knowledge, this *in vitro* study is the first to demonstrate the alteration of leptin pathway after NDL PCB exposure (PCB 101, PCB 153 and PCB 180), indicating a detrimental effect of these pollutants on adipocyte function and activity. The relevance of these findings concerns the metabolic detrimental effects shown by three representative PCBs among the six PCB indicators, whose "sum is considered as an appropriate marker for occurrence and human exposure to NDL-PCB" (verbatim reported from EU Commission Regulation 1259/2011). Among these iPCBs, we choose the most frequently detected at high residue levels in human tissues, including adipose tissue (Corsolini et al., 1995; Duarte-Davidson et al., 1994; Malarvannan et al., 2013).

Obesity arises from abnormal growth of adipocytes leading to increased total fat mass. Indeed, pathological adipocyte alteration confers abnormal expression of adipocytokines, which are implicated in insulin resistance and metabolic disorders. Among adipokines, leptin, synthesized and released proportionally to fat mass content (Kallen and

Lazar, 1996), plays a pivotal role in maintaining adipose tissue balance/stabilization. Indeed, leptin, through the binding of its functional receptor isoform on adipocytes, activates a signalling cascade, leading to an increase in fatty acid oxidation, and hence a lipostatic effect (Lopez et al., 2007b). The alteration of this homeostatic pathway, characterized by an increase in circulating leptin levels and decreased leptin receptor signalling, is defined leptin resistance, and often associated to the obese phenotype (Zhou and Rui, 2013).

Here, we have demonstrated that NDL PCB exposure increased lipid content in mature adipocytes, disrupting leptin sensitivity through the downregulation of its functional receptor and signalling transduction. We also evidenced that leptin transcription was increased in 3T3-L1 cells after PCB exposure, further confirming the onset of leptin resistance. Our results are consistent with those by Taxvig et al. (2012) which evidenced, an increase in leptin release and lipid accumulation in 3T3-L1 adipocytes treated with PCB 153. Other researchers demonstrated that *in vivo* PCB 153 administration in animals on high-fat diet, significantly increases leptin plasma levels and disrupts hepatic lipid metabolism (Wahlang et al., 2013).

Leptin is considered an anti-obesity hormone, able to prevent the accumulation of lipid, thereby preventing the functional impairment referred to lipotoxicity (Nedergaard et al., 2001). Indeed, leptin promotes partitioning of metabolic fuels toward utilization rather than storage, decreasing the incorporation of fatty acids into triglycerides and increasing fatty acid oxidation (Rahmouni and Haynes, 2001; Muoio et al., 1997; Steinberg et al., 2002).

When leptin resistance occurs, the decreased sensitivity to the metabolic effect of leptin leads to an accumulation of lipids in adipose and non-adipose tissues, through the

inhibition of AMPK/ACC pathway (Minokoshi et al., 2002). In our experimental condition, the increase in leptin synthesis and the reduction in leptin receptor transcription by PCBs is also associated to the reduction of phosphorylated enzymes AMPK and ACC, and hence the increase in lipid content in adipocytes.

Leptin effect on adipose tissue is orchestrated by activation/phosphorylation of AMPK. This is a nutrient-sensitive kinase, playing a pivotal role in mammalian energy metabolism (Unger, 2004; Friedman and Halaas, 1998), monitoring cellular energy status, through the sensing of [ATP]/[AMP] ratio. When the [ATP]/[AMP] ratio is lowered due to reduced nutrient availability, AMPK is activated. Therefore, activation of AMPK represents a signal to shut down anabolic pathways and to promote catabolic processes phosphorylating key enzymes of intermediary metabolism. In parallel with AMPK activation, leptin suppresses ACC activity, thereby lowering malonyl-CoA and disinhibiting carnitine palmitoyltransferase (CTP) 1. This latter effect leads to fatty acids transport into the mitochondria and hence their β-oxidation. Therefore, it is conceivable to argue that PCB induction of leptin resistance might be related to the increase of fat accumulation by modulating AMPK/ACC pathway in adipocytes.

To confirm PCB-induced leptin resistance, we evaluated the effectiveness of leptin signalling through the evaluation of phosphorylated STAT3 and the expression of the phosphatase PTP1B, an important mediator of leptin resistance (Cheng et al., 2002; Zabolotny et al., 2002). In particular, PTP1B negatively regulates leptin signalling by dephosphorylating JAK2, and hence reducing its phosphorylating activity on STAT3 (Cheng et al., 2002; Zabolotny et al., 2002). Consistently, we found an increase in PTP1B expression and a reduction in the activation of STAT3 in PCB treated cells, showing PCB capability to impair leptin sensitivity.

In addition to the metabolic actions of AMPK, increasing evidence suggests that AMPK has anti-inflammatory actions, independently from its effects on lipid metabolism (Salt and Palmer, 2012). Indeed, although the role of AMPK in liver and muscle is relatively well characterized and has been extensively studied, the role of AMPK in other metabolic tissues, as AT, remains undefined. Dysfunctional metabolism and a chronic low grade of inflammation are observed in AT of obese individuals, contributing to metabolic impairments often associated to an increase in inflammatory mediators or cytokines. Lihn et al. (2008) has reported that activation of AMPK can inhibit the synthesis of pro-inflammatory cytokines, such as IL-6 and IL-8 in adipose tissue. Here, the ability of PCBs to down-regulate AMPK activation is consistent with the observed increase in TNFα and IL-6 synthesis. As well known these two cytokines, produced by adipose tissue, are secreted into the circulation, and their elevation plays a role in the onset of metabolic dysfunction related to type 2 diabetes, or metabolic syndrome (Wieser et al., 2013; Capurso and Capurso, 2012).

It is worthy to note that the PCB concentrations used in the current study were comparable to those observed in epidemiological studies in adipose tissue and other biological animal and human samples (Duarte-Davidson et al., 1994; Ferrante et al., 2007).

In our study we observed a synergistic effect of NDL PCB tested both on metabolic impairment (i.e. leptin and leptin receptor transcription, and leptin signalling) and inflammatory cytokines (i.e. IL-6 and TNFα). These data are more relevant compared to those obtained from PCB alone, since humans are always exposed to complex mixtures of these pollutants, leading to synergistic or additive effect, most likely on the basis of the chlorine substitution and/or different mechanisms of action. The differential effects induced by the PCB congeners might be explained with a congener specific

responsiveness, but also with a heterogeneity existing on the basis of the distribution of PCBs across adipocyte compartments.

In conclusion, our findings suggest that disruption of lipid metabolism is caused by PCBs exposure in 3T3-L1 differentiated cells through the reduction of leptin receptor responsiveness and its related pathways. This study shows the potential obesogenic effect of these NDL PCBs, that are prevalent in the environment contributing to metabolic and inflammatory disorders. NDL-PCBs are, therefore, POPs that accumulate preferentially in adipose tissue causing a low grad inflammation and endocrine disruption.

Obesity is a clear risk factor for OA, whose pathogenesis has been recently associated with adipokines (Conde et al., 2011; Scotece et al., 2013; Otero et al. 2005). Our above mentioned results, in according with other *in vitro* studies, have shown that NDL-PCBs upregulated leptin levels, probably leading to an obesogenic effect (Wahlang et al., 2013; Taxvig et al., 2012). However, only few studies have analyzed the correlation between exposure to PCBs and pathogenesis of OA. Intriguingly, most of this evidence have highlighted the presence of a strongly positive correlation between high serum levels of PCBs, as a consequence of environmental poisoning, and the incidence of swelling of the joints and arthralgia (Kanagawa et al., 2008), as well as of OA (Guo et al., 1999), mainly in women (Lee et al., 2007).

Moreover, destruction of the cartilage matrix by a pathological imbalance of normal chondrocyte function is a key step in OA progression. Chondrocyte is the only cell in articular cartilage and plays an essential role for its integrity. Thus, chondrocyte cell death leads, as logic consequence, to cartilage damage. Indeed, chondrocyte apoptosis is frequently observed in advanced cases of OA (Heraud et al., 2000).

It has been reported that NDL-PCBs induce apoptosis in several cell lines (Ferrante et al., 2011; Ghosh et al., 2010; Sánchez-Alonso et al., 2003; Shin et al., 2000). Recently, Lee and Yang (2012) have demonstrated that the DL-PCB 126 exerts an apoptotic effect in chondrocytes, even if *in vitro* and mechanistic studies about NDL-PCB-driven cell death are not currently available.

To the best of our knowledge, our study for the first time shows the concentration dependent reduction of murine ATDC5 and human T/C-28a2 chondrocyte viability by PCBs 101, 153, and 180 and the involvement of two processes in controlling of cell death: apoptosis and necrosis.

As is well known, these processes include a spectrum of partially overlapping cellular events (Kühn et al., 2004). Thus, special care was taken to gain insights into the mechanisms of cell death elicited by PCBs by using different methods. In ATDC5 cells, both LDH and Annexin V-FITC/propidium iodide (PI) assays showed that all the PCBs analyzed induced necrosis in a concentration-dependent manner, but the latter assay highlighted also their capability to induce late apoptosis, although in a slight manner.

In order to assess the relationship between PCB and chondrocyte apoptosis, we evaluated caspase 3 activation. Indeed, the activation of this pathway, by proteolitic cleavage, is considered a break point in apoptosis, resulting an useful marker of its induction. Our data demonstrate that all PCBs tested significantly increase cleaved-caspase-3 expression in a concentration dependent manner, suggesting that also apoptosis contributed to NDL-PCB driven cell death in chondrocytes.

Apoptosis in mammalian cells is mediated by the intrinsic and the extrinsic pathways. The intrinsic pathway, involving cytochrome c and caspase activation, is regulated by members of the Bcl-2 family (Elmore, 2007; Cory and Adams, 2002).

In particular, two counteracting proteins, the anti-apoptotic Bcl-2 and the pro-apoptotic Bax, play a key role in the intrinsic pathway activation (Oltvai et al., 1993). When Bcl-2 is present in excess, cells are protected against apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed death. In this study, we observed an altered expression of Bcl-2 and Bax in ATDC5 chondrocytes incubated with all pollutants tested. To note, the Bcl-2/Bax ratio decreased after 24 h PCB exposure in a concentration-dependent manner, suggesting that the intrinsic pathway of PCB-driven apoptosis was also in play. Our findings are in agreement with previous results by Santiago et al. (2006) and Ferrante et al. (2011). In this latter study, in particular, it has been demonstrated the involvement of the intrinsic pathway in NDL-PCB 101, 153 and 180-induced apoptosis in J774A.1 murine macrophages.

Earlier studies have demonstrated that the p38 activation can lead to a Bax increased expression, Bcl-2 down-regulation and cytochrome c release into the cytoplasm (Kong et al., 2013; Wang et al., 2007; Zhuang et al., 2000). It is noteworthy that our results showed that all PCBs tested so far increased p38 phosphorylation in a concentration-dependent manner, in agreement with the reduction of Bcl-2/Bax.

Oxidative stress is also a major mechanism involved in the pathogenesis of OA (Alcaraz et al., 2010; Sutipornpalangkul et al., 2009). Since oxidative stress damages mitochondrial DNA, resulting in apoptosis and senescence of chondrocytes (Henrotin et al., 2010), we evaluated its involvement in NDL-PCB induced apoptosis. Interestingly, our results show that these pollutants increase MDA levels and OSI in these cells in a marked concentration-dependent manner. In agreement with above mentioned results, the TAS, a

parameter of antioxidant defences [i.e., superoxide dismutase (SOD), catalase (CAT), glutathione (GSH)], is significantly decreased after PCB treatment.

Therefore, our study demonstrate for the first time that NDL-PCBs are able to induce apoptosis in chondrocytes, probably resulting in cartilage degradation related to OA. Although our findings should be validated by in *in vivo* experiments, it is plausible to assert that NDL-PCBs might play a role in the pathogenesis and ongoing of this pathology.

These last results further confirm the hypothesis of "obesogenic" role of these pollutants and their involvement not only in metabolic impairment and obesity but also in their related pathologies.

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