

An integrated approach to decipher the chemical exposome of women: application to etiological research of metabolic and reproductive disorders

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Par
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An integrated approach to decipher the chemical exposome of women: application to etiological research of metabolic and reproductive disorders

Travaux à présenter et soutenir à Nantes, le 31 de Janvier de 2024
Unité de recherche : LABERCA (UMR 1329)

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Résumé

Les humains sont exposés à un nombre croissant de substances chimiques synthétiques qui peuvent interagir avec les systèmes biologiques et, par conséquent, être impliqués dans la pathogenèse de multiples maladies. L'identification des associations causales entre cette exposition chimique et la santé humaine reste un défi très complexe, en particulier dans la recherche observationnelle, en raison de la multiplicité des expositions chimiques, de la présence d'effets de mélange et non linéaires, d'effets à long terme issus d'exposition précoce, entre autres problématiques. Les avancées technologiques en spectrométrie de masse ont fait émerger le développement de nouvelles stratégies pour l'évaluation de l'exposition chimique, mais aussi pour la caractérisation de composés endogènes susceptibles d'éclairer les mécanismes biologiques liés aux maladies (biomarqueurs d'effet). Les méthodes statistiques d'analyse de données ont également considérablement évolué au cours de la dernière décennie pour s'adapter à un grand nombre de biomarqueurs et des structures d'association complexes. Ces avancées ont contribué à façonner le concept d'exposome et son application dans différents domaines de la recherche en santé environnementale. Le concept holistique d'exposome offre ainsi aujourd'hui de nouvelles opportunités pour combler les lacunes de connaissances autour du rôle des produits chimiques environnementaux dans l'étiologie de certaines pathologies, et en particulier dans le cadre de nos travaux autour des troubles reproductifs féminins complexes et multifactoriels tels que l'endométriose ou l'infertilité.

Ce document est structuré en quatre sections principales : 1) Contexte, 2) Contributions scientifiques, 3) Perspectives de recherche scientifique et 4) Candidat et production scientifique.

La première section, présente des familles de produits chimiques synthétiques d'intérêt pour leur présence ubiquitaire et bio-activité, suivie d'un aperçu du contexte réglementaire Européen de l'évaluation de risques chimiques. Le processus d'évaluation de l'exposition chimique en mettant l'accent sur l'exposition au cours de la première période de la vie est ensuit introduit. Finalement quelques questions méthodologiques liées au lien entre les expositions chimiques et la santé sont discutés, y compris l'évaluation des effets des mélanges ou l'intégration des biomarqueurs d'effet dans l'épidémiologie environnementale.

La deuxième section résume les principaux résultats de mes recherches visant à mieux caractériser l'exposome chimique et mieux comprendre ses relations avec la santé. Ce bilan reflète les travaux conduits et résultats produits depuis la fin de mon deuxième post-doctorat à l'Université de Californie à Davis et la période d'intégration dans mon unité d'accueil le LABERCA UMR1329 ONIRIS-INRAE (depuis octobre 2016). Cette section ne suit pas un ordre chronologique, mais a été structurée thématiquement selon les principales fenêtres critiques d'exposition chimique au cours de la vie. Un premier chapitre est ainsi axé sur l'exposition périnatale et ses impacts sur la programmation du surpoids/obésité de l'enfant, incluant une approche *in vitro* et deux approches épidémiologiques novatrices. Un deuxième chapitre traite des recherches sur l'exposition chimique interne des femmes en âge de procréer et ses impacts sur les troubles de la reproduction, tels que l'endométriose, la fonction ovarienne et les résultats de la fécondation in vitro. Le dernier chapitre présente les résultats des recherches sur l'exposition chimique des femmes âgées et ses associations avec le risque de présence et d'agressivité du cancer du sein.

La troisième section présente les perspectives de recherche scientifique issues de ce bilan et de cette trajectoire de recherche, construites sur la base de projets de recherche en cours déjà financés et sur de nouveaux projets en cours d'évaluation ou de montage, alignées sur les missions de l'institut et de l'unité d'accueil.

La dernière section présente le profil du candidat, ses activités de production scientifique et d'enseignement, ainsi que l'unité d'accueil, donc la capacité personnelle et logistique pour développer le programme de recherche proposé.

Summary

Humans are exposed to a growing list of synthetic chemicals that can interact with biological systems and thus, being involved in pathogenesis of multiple diseases. Nevertheless, identifying causal associations between chemicals and human health remains very challenging especially in observational research due to the multiplicity of chemical exposures or the presence of mixture and non-linear effects, long-term effects due to early and lifespan exposure, among other issues. Technological advances in mass-spectrometry have favored the development of novel strategies for chemical exposure assessment, but also, for the characterization of endogenous compounds with the potential of informing biological mechanisms linking with diseases (e.g. effect biomarkers). In addition, the statistical methods have substantially evolved during the last decade to accommodate large number of biomarkers and complex causal structures. These advances have contributed to shape the "exposome" concept and its operationalization in different domains of environmental health research. This new holistic exposome concept provides novel opportunities to fill longstanding gaps arising the role of environmental chemicals in the etiology of complex and multifactorial female reproductive disorders such as endometriosis or infertility.

This document has four main sections: 1) Context, 2) Scientific contributions, 3) Scientific research perspectives and 4) Candidate and scientific production.

In the first section, the major families of synthetic chemicals of concern for human health are presented. Then, an overview of the European chemical regulatory context and the chemical exposure assessment process is provided, with a focus about the exposure during the first period of life. Thereafter, common methodological issues including those related to the assessment of mixture effects or integration of effect biomarkers in environmental epidemiology are introduced.

The second section summarizes the main results of selected recent research projects where I have contributed to gain insight into the synthetic chemical exposome and its relationships with health. This period reflects the results since the end of my second postdoctoral scholar at the University of California at Davis and the integration period in my hosting unit LABERCA UMR1329 ONIRIS-INRAE (since October 2016). This section does not follow a chronological ordering; instead, the contributions have been structured in main critical windows of chemical exposure during the lifespan with a focus on female health. Hence, a first chapter is focused on the perinatal exposure to environmental chemicals and its impacts on overweight/obesity programming, including a cell-based and two exposome-scale epidemiological approaches. A second chapter elaborates the research on internal chemical exposure of women at reproductive age and its impacts on reproductive disorders, such as endometriosis, ovarian function and *in vitro* fertilization outcomes. The final chapter shows the results of research about the chemical exposure of elderly women and its associations with the risk of breast cancer presence and aggressiveness.

The third section presents the scientific research perspectives built on the on-ongoing research projects, already granted, and submitted proposals under evaluation, aligned with the missions of hosting institute and unit.

The final section presents the academic profile, the scientific production and teaching activities, as well as the hosting unit, hence the personal and logistic capacity to develop the proposed research program.

List of abbreviations

ANSES, (French abbreviation of French Agency for Food, Environmental and Occupational Health & Safety)

AOP, adverse outcome pathway

BFR, brominated flame retardant

BKMR, Bayesian Kernel Machine Regression

CAGR, compound annual growth rate

DDT, dichlorodiphenyltrichloroethane

DL, dioxin-like

ECHA, European Chemicals Agency

EFSA, European Food Safety Authority

HBCD, hexabromocyclododecanes

HCB, hexachlorobenzene

HRMS, high-resolution mass spectrometry

HTS, high-throughput screening

IARC, International Agency for Research on Cancer

IVF, in vitro fertilization

MITM, meet-in-the-middle

NDL, non dioxin-like

OCPs, organochlorine pesticides

OECD, Organization for Economic Cooperation and Development

OPFR, organophosphate ester flame retardants

PBBs, polybrominated biphenyls

PBDEs polybrominated diphenyl ethers

PCBs, Polychlorinated biphenyls

PCDDs, polychlorinated dibenzo-para-dioxins

PCDFs, polychlorinated dibenzofurans

PFAS, per-/polyfluoroalkylated related substances

PFOA, perfluorooctanoic acid

PFOS, perfluorooctane sulfonic acid

POPs, Persistent Organic Pollutants

REACH, Registration, Evaluation and Authorization of Chemicals

SVHC, substance of very high concern

TBBP-A, tetrabromobisphenol A

TPhP, triphenyl phosphate

TSCA, Toxic Substances Control Act

US-EPA, United States Environmental Protection Agency

WHO, World Health Organization

β-HCH, β-hexachlorocyclohexane

1. CONTEXT

1.1 Introduction to synthetic chemical pollutant

The chemical industry has played an unquestionable role in modern human lives as anticipated in the early book of Abraham Cressy Morrison "Man in a chemical world " early in 1937 (Homburg and Travis 1998). The Second Industrial Revolution (1850-1914) provided a number of technological advances that boosted the chemical industry development, placing Europe in the center of modern international chemical industry development. The geopolitical scenario favored the convergence of science and technology, boosting the innovations on synthetic dyestuffs (e.g. aniline dye) and pharmaceuticals. The period between 1856-1918 was also highlighted by the heavy production of artificial fertilizers and sulphuric acid. Sulphuric acid was also extensively used in pest control up to the 1940s with other inorganic substances such as sodium chlorate.

Agrochemicals

Some pesticides were by-products of coal gas production or other industrial processes, such as nitrophenols, chlorophenols, creosote, naphthalene and petroleum oils, used for fungal and insect pests, whilst ammonium sulphate and sodium arsenate were used as herbicides. The growth in synthetic pesticides accelerated in the 1940s with the discovery of the effects of technical dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), aldrin, dieldrin, endrin, chlordane, parathion, captan and 2,4-dichlorophenoxyacetic acid (2,4-D). These organochlorine pesticides (OCPs) gained popularity because were effective and inexpensive, specially DDT duet to its broad-spectrum activity. Hence, DDT was one of the most widely used organochlorine insecticides, also because appeared to have low toxicity to mammals, and reduced insect-born diseases, like malaria, yellow fever and typhus. The popularity of DDT declined since 1946 when house flies exhibited resistance due to its overuse, in addition of growing reports of harm to non-target plants and animals or raising issues with residue management. The concern of organochlorine agrochemicals was reinforced by their toxicity, high persistence and mobility in the environment, in addition to the capacity to bioaccumulate within individuals and bio-amplify across species in the trophic chain. Most of these chemicals are considered semi-volatile organic compounds are subject to Long-Range Atmospheric Transport, occurring occur through a series of deposition–reemission events at the soil–air interface and thus being found in remote areas far from emission sources (Loizeau et al. 2014). Consequently, most of them were banned since early 1970s in western countries and matter of concern of international committees due to its global effects. Aldrin, chlordane, dieldrin, endrin, heptachlor, HCB, mirex, toxaphene and DDT were among the first 12 chemicals listed as Persistent Organic Pollutants (POPs) in the Stockholm Convention in 2000, thus their production ceased before the 2000s in most countries. Whereas most organochlorine pesticides were listed in Annex A to prompt their worldwide elimination, DDT was reintroduced by the World Health Organization (WHO) in 2006 and listed in Annex B intending to regulate its applications to control mosquito-borne diseases, such as malaria, because remained the unique successful strategy (van den Berg et al. 2021). Hence, the global production of DDT in 2014 was estimated at 3491 metric tons of active ingredient, being India the largest producer and user of DDT (van den Berg et al. 2017). The use of the organochlorine DDT at global level decreased by 62.0% during the study period (2010-2019), with 13 countries reporting DDT use in 2010 and five countries still using DDT in 2019. In addition, HCB is still being released to the environment, 70%–95% lower than that in 1970, as an unintended byproduct in chemical processes, incomplete combustion and an impurity in pesticides (Wang et al. 2010). In 2009, four organochlorine pesticides, chlordecone, alpha hexachlorocyclohexane, beta hexachlorocyclohexane, lindane, pentachlorobenzene were added to the list (*UNEP* 2013, 2019).

To overcome the bans, research into new pesticides continued and the 1970s and 1980s brought the introduction of the world's greatest selling herbicide, glyphosate, imidazolinone herbicides, and the introduction of dinitroanilines, aryloxyphenoxypropionate and cyclohexanediones families. During that time also emerged a 3rd generation of pyrethroids, the introduction of avermectins or

benzoylureas. This period also saw the introduction of the triazole, morpholine, imidazole, pyrimidine and dicarboxamide families of fungicides. As many of the agrochemicals introduced at this time had a single mode of action, thus making them more selective. In the 1990s research activities concentrated on finding new members of existing families which have greater selectivity and better environmental and toxicological profiles. In addition, new families of agrochemicals have been introduced to the market such as the triazolopyrimidine, triketone and isoxazole herbicides, the strobilurin and azolone fungicides and chloronicotinyl, spinosyn, fiprole and diacylhydrazine insectides. Many of the new agrochemicals could be used at grams rather than the kilograms per hectare. The global agrochemicals market size was valued at USD 224.9 billion in 2021 and is anticipated to expand at a compound annual growth rate (CAGR) of 3.2% from 2022 to 2030. The growth is attributed to the increasing demand for fertilizers and crop protection products in the field of agriculture across the globe. The increasing use of chemical fertilizers by farmers to supply nutrients to the crops and enhance their yield is a key factor driving the agrochemicals demand.

Industrial compounds and by-product of industrial processes

Polychlorinated biphenyls (PCBs) are a family of 209 synthetic chemicals consisting of two phenyl rings in the same plane with coplanar configuration (*non-ortho* PCBs) or non-coplanar (*ortho*-substituted PCBs) (Figure 1). PCBs were first described in de 1880s and manufactured industrially since 1929 boosted by Montanto's Aroclor® products and the applications of PCBs during the World War II. After the war, the applications areas extended to flame retardant heat transfer fluids, hydraulic fluids, and plasticizers and heavily produced until late 1970s, yet many products remained in the market decades after the phase-out (Erickson and Kaley 2011).

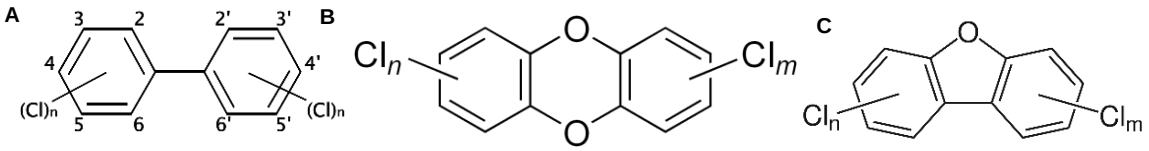


Figure 1. General chemical structure of PCBs (A), PCDDs (B) and PCDFs (C)

The polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two series of almost planar tricyclic aromatic compounds with very similar chemical properties (Arisawa et al. 2005; *UNEP* 2013). PCDDs and PCDFs are not commercially produced but are formed as trace amounts of undesired impurities in the manufacture of other chemicals, such as chlorinated phenols and their derivatives, chlorinated diphenyl ethers and polychlorinated biphenyls (PCBs). Ongoing or earlier use of pentachlorophenol is considered to be a major source of PCDDs and PCDFs in many industrialized countries.

Flame retardants (1) - brominated

Bromine was discovered in 1826 by Antoine Jerome Balard (1802-1876) a young student of pharmacy at the Faculté des Sciences in Montpellier (Wisniak 2002), but the bromine industry ban in 1865 when the halogen was successfully isolated. The needs for bromine developed very slowly and it took almost a century, until World War I (WWI), to achieve the status of a major chemical commodity. The production of bromine was boosted by the development of the automobile industry, specially by the use of ethylene dibromide as antiknock additive for diesel engines, hence the production in 1925 already reached 6000 tons/year. At that time, in 1930 ethylene dibromide and ethyl bromide were found to be efficient grain fumigants, hence the diversification of bromine started including further application in the baking and brewing industries and more recently flame extinguishing and flame proofing. In 1999 the world bromine consumption was already dominated by the use in flame retardants (27%), followed by manufacture of ethylene dibromide (15%) or agricultural chemicals (15%). The use of bromine in flame retardant applications raised in the 1970s, boosted in the United States and Canada by the enforcement of flammability standards such

as the Californian Technical Bulletin 117 (TB 117) in 1975 to protect against home fires started by small open flames, such as candles, matches, and lighters (Stapleton et al. 2012). Major brominated flame retardants include polybrominated diphenyl ethers (PBDEs, phased out in 2002), hexabromocyclododecanes (HBCD) and tetrabromobisphenol A (TBBP-A), being mainly produced in Asia (Law et al. 2006):

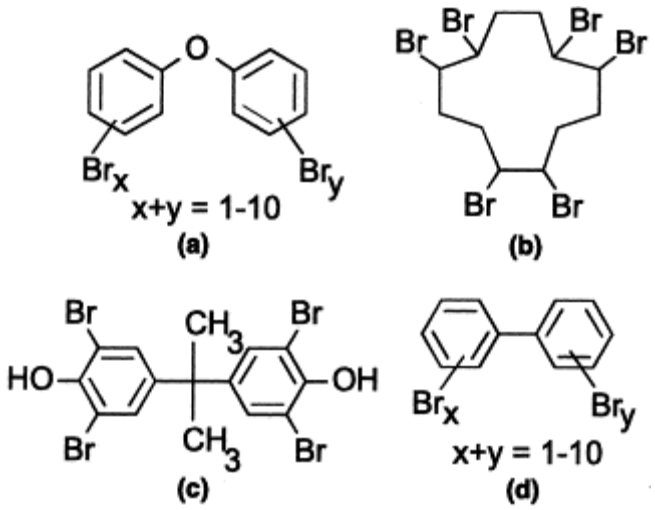


Figure 2. The chemical structures of PBDEs (A), HBCD (B), TBBPA (C), and PBBs (D) (de Wit 2002).

The PBDEs involves 209 possible congeners with ten hydrogen atoms (Figure 2 A), have been extensively used as non-reactive additives in textiles, polyurethane foams, thermoplastics, and electronic products, details in Table 1 (de Wit 2002; Hale et al. 2002). Noteworthy, natural production of BDEs by proteobacteria, algae or marine sponges has also been described (Agarwal et al. 2017; Shaw and Kannan 2009), bioaccumulating along the trophic chain and thus reaching human diets with synthetic isoforms (Wan et al. 2010). In 2001, EU announced a ban on Penta- and Octa-PBDE formulations by 2003. In 2002, the industry voluntarily banned the production of Penta and Octa formulations as of 2004. Additionally, since July 1, 2006, Penta and Octa-BDE were banned in electrical and electronic applications according to the 2002/95/EC directive.

Table 1. Uses, composition and consumption of the three common commercial BDE products (Bogdal et al. 2014; Hale et al. 2002)

Commercial product	Common usage	Major constituents of structure	Maket Demand	
			Total global (in metric tons)	Percent of total used in North America (%)
Penta- (phased out 2004, SC)	Polyurethane foam – In furniture	BDE47 BDE99 BDE BDE153 BDE154	8500	98
Octa- (phased out 2004, SC)	Rigid plastics – In housings for electronics	Specific constituents unknown, consists of 70–80% hepta- and octa-congeners	3825	36
Deca- (2012, SVHC and SC)	Rigid plastics, resins and coatings – In housings for electronics, textiles, insulators	BDE-209	54800	44

The Persistent Organic Pollutants Review Committee recommended listing and specifying the related control measures of the decabromodiphenyl ether component (BDE-209) of c-decaBDE

in Annex A with a specific exemptions (*UNEP* 2013, 2019). Technical decaBDE may include large proportions of lower brominated congeners among the impurities. The global in-use stock of PBDEs in industrialized regions increased with doubling times from 7 to 12 years from the late 1970s to early 2000s (Abbasi et al. 2019). Current sources of PBDES may include the inadvertent impact of recycling that has resulted in peak emissions of those legacy compounds in plastic compounds including children toys (Ionas et al. 2014).

The HBCD has been one of the most widely used aliphatic cyclic additive BFR with a total global market value between 15,900 and 21,951 metric tons in 1999 and 2003, respectively. HBCD is used in construction and insulation boards, packaging material, electrical and electronic equipment, upholstered fabric and textiles, bed mattress, furniture, seating, draperies, wall coverings, indoor textiles and automobile indoor textiles. The European market demand for HBCD in 2001 was 9,500 metric tons, which accounts for 57% of the global market demand. Normally, the γ-isomer is the most dominant in the commercial mixtures (ranging between 75% and 89%), followed by α- and then β-isomer (10–13% and 1–12%, respectively). The Stockholm Convention on POPs decided in May 2013 to list hexabromocyclododecane in Annex A to the convention with specific exemptions for production and use in expanded polystyrene and extruded polystyrene in buildings (*UNEP* 2013, 2019).

TBBPA is currently the most widely used brominated flame retardant (BFR) with an annual demand of about 130,000 metric tons in 2002 of which approximately 85% are used in East Asia. The global TBBPA market is expected to grow from USD 1.02 billion in 2017 to USD 1.48 billion by 2030, at a CAGR of 4.5% from 2017 to 2030. The industrial grade TBBPA are widely used in electrical and electronics industry as a flame retardant. The bromobisphenol A (BBPA) and TBBPA-bis(2,3-dibromopropyl ether) (TBBPA-BDBPE) are considered high-production-volume chemicals by the Organization for Economic Cooperation and Development (OECD) (3,4) and the United States Environmental Protection Agency (US EPA). (5)

The PBBs belong to a class of halogenated biphenyls where 1–10 hydrogen atoms are replaced with bromine atoms also involving 209 congeners as PBDEs (Figure 2D). PBBs were manufactured in the early 1970s for commercial use and consist mainly of hexa-, octa-, nona- and deca-bromobiphenyl congeners. PBBs came to public attention in 1974, when it was discovered that about 1,000 pounds was accidentally substituted by magnesium oxide, an additive in cattle feed, in Michigan in 1973, prompting the subsequent progressive cease of production completed in 2000. In Europe, PBBs were restricted by the fourth amendment to the marketing and use Directive 76/769/EEC adopted in 1984, and they cannot be used in textiles, intended to come into contact with the skin.

Flame retardants (2) - Organophosphate Ester

Organophosphate ester flame retardants (OPFR) are organic esters of phosphoric acid-containing either alkyl chains or aryl groups, and they may be halogenated or non-halogenated, also used as plasticizers in consumer products and construction materials. Commercial flame retardant formulations that contain mainly OPFRs have increasingly replaced PentaBDE in residential furniture (Cooper et al. 2016). Different commercial mixtures of flame retardants contain organophosphate esters, including Firemaster 550 introduced in 1995 in the US. The exact composition of those mixtures chemicals is protected by intellectual and commercial property rights, yet it is known the presence of compounds such as triphenyl phosphate (TPhP), isopropylated TPhP isomers, 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (TBB) and tetrabromophthalate (TBPH). The use of organophosphate ester flame retardants as substitutes of BDEs has been questioned by the scientific community due to their potential human health effects and widespread dissemination in the environment (Blum et al. 2019). Out of the total consumption of flame retardants in Europe during 2015, 18% were phosphorus flame retardants, representing

89,640 metric ton, consistently found in dust and human biological samples from European countries, being TPhP, isopropylated triarylphosphate isomers (ITPs) and tris(1-chloro-2-propyl)phosphate (TCIPP) the most concerning in terms of occurrence and potential health effects (Chupeau et al. 2020)

Fluorinated chemicals

Fluorinated polymers are made up of one or several types of monomer blocks containing carbon-fluorine bonds in their backbones and/or side chains. The history of fluoropolymers began by the accidental production of polytetrafluoroethylene (PTFE) from refrigerant gases in 1938. PTFE is inert to virtually all chemicals and considered the most slippery material in existence, making it one of the most valuable, versatile technologies invented later registered under the trademark of Teflon™ by DuPont. Since then, more than 4,730 per- and polyfluoroalkylated related substances (PFAS) have been identified in recent inventories with known CAS numbers, being most of them (~4186) precursors in environment or biota (McDonough et al. 2022) .

The unique properties have prompted their extensive use in water-, soil-, and stain-resistant coatings for clothing, leather, upholstery, and carpets; oil-resistant coatings for food contact paper; aviation hydraulic fluids; fire-fighting foams; paints, adhesives, waxes, polishes, and other products; and industrially as surfactants, emulsifiers, wetting agents, additives, and coatings. The most relevant perfluoroalkyl carboxylic acid is the perfluorooctanoic acid (PFOA), whereas perfluorooctane sulfonic acid (PFOS) has become the most heavily produced perfluoroalkane sulfonic acid, both by the 3M company until 2002, when were phased-out (Land et al. 2018). The 3M production of PFAS was dominated by electrochemical-fluorination, production a mixture of linear and branched isomers (at 70:30 rate). Since then, manufacturers have been developing replacement technologies, including reformulating or substituting longer-chain substances with non-fluorinated chemicals, alternate technologies, or shorter-chain perfluoroalkylated or polyfluorinated substances. Some alternate PFAS include, but are not limited to, compounds produced with ECF and fluorotelomerization, such as: FTOH, perfluorobutane sulfonyl fluoride (PBSF)-based derivatives (p.e. perfluorobutane sulfonate [PFBS] in lieu of PFOS), per- and polyfluoroalkylethers (p.e. GenX and ADONA) and other types of PFAS. Moreover, Asian manufacturers (e.g. Rusia, India or China) began in the 2000s the large-scale production of long-chain PFAS and their precursors, using fluorotelomerizaton that result in 100% of lineal chains. Despite the pressure over fluoropolymer industry, the trends of production does not seems to decline with an annual growth rate (CAGR) of 5.6% during 2016–2022 ([Anonymous] 2017; Améduri 2020) REF, being the Asia-Pacific region the main producers and North America the main consumers (40% global production in 2014) and an estimated emission to the environment of 75000 tons in 2020. The 7th February 2023 a novel restriction proposal prepared by authorities in Denmark, Germany, the Netherlands, Norway and Sweden has been published by the European Chemicals Agency (ECHA) to regulate the use of around 10000 PFAS with the intend of reducing the release of 4.4M tons over the next 30 years.

Chemical plasticizers and additives

The first known polymer with plastic properties was synthesized by Alexander Parkes in 1862, however the invention of cellophane in 1913, then polyvinyl chloride in 1927, polystyrene and nylon in 1938, and polyethylene in 1942 were the main milestones that established the foundations of massive growth increase of plastic industry. Actually, the plastic production around the world has grown faster than that of any other material since the 1950s ; 230-fold from 2 million tons in 1950 to 460 Mt in 2019 (OCDE 2022; The Lancet Planetary 2018). This has largely been due to the increasing manufacture of plastic products that are meant to be discarded after one use, so-called single-use plastics. Packaging is the main application of worldwide plastic production, followed by building and construction, vehicles, consumer product or clothing. The production of plastic often requires additives known as plasticizers, relatively non-volatile organic substances

incorporated into to improve the polymer's flexibility, extensibility and processability. There are about 30 000 chemicals can be potentially utilized as plasticizers, including phthalates, trimellitates, adipates or organophosphate classes. Bisphenols, wrongly labelled as additives, are intermediates in the production of polycarbonate plastics and epoxy resins. Epoxy resins are used as food-contact surface coatings for cans, metal jar lids, coatings and finishes, automobile parts, adhesives, aerospace applications, and as a coating for polyvinyl chloride (PVC) water pipe walls. Polycarbonate plastics are hard plastics used to make numerous products, such as eyeglass lenses, water bottles, and consumer electronics.

1.2 Chemical risk assessment regulatory context

Since mid-1900s, chemical risk assessment has emerged in parallel to the chemical industrial revolution as a structured and integrated process to ensuring the safety of humans in front of the adverse health effects derived from chemical exposure. Despite there is evidence that Romans were already aware and took actions against lead poisoning, the health impacts of modern chemistry began nearly 200 years ago. For instance, the first evidence of dioxin production was documented in 1827 in German chemical production plant in Lampertheim. Nonetheless, during the beginning of 20th century, the incipient growth of chemical industry was not seen as a sign of concern but of well-ness development until some large-scale chemical health issues like Minnamata disease (1956) began uncovering the tip of the iceberg. During that time, the chemical "safe dose" concept, early introduced by Paracelsus (1500s), gained a prominent position in the emergence of benchmarking safe level process integrated by National Institute for Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) in United States, but also Council of Europe in 1962 and later adopted the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Major historical milestones in chemical risk assessment include the creation of national and international agencies and task forces dedicated to chemical risk assessment throughout robust scientific methods.

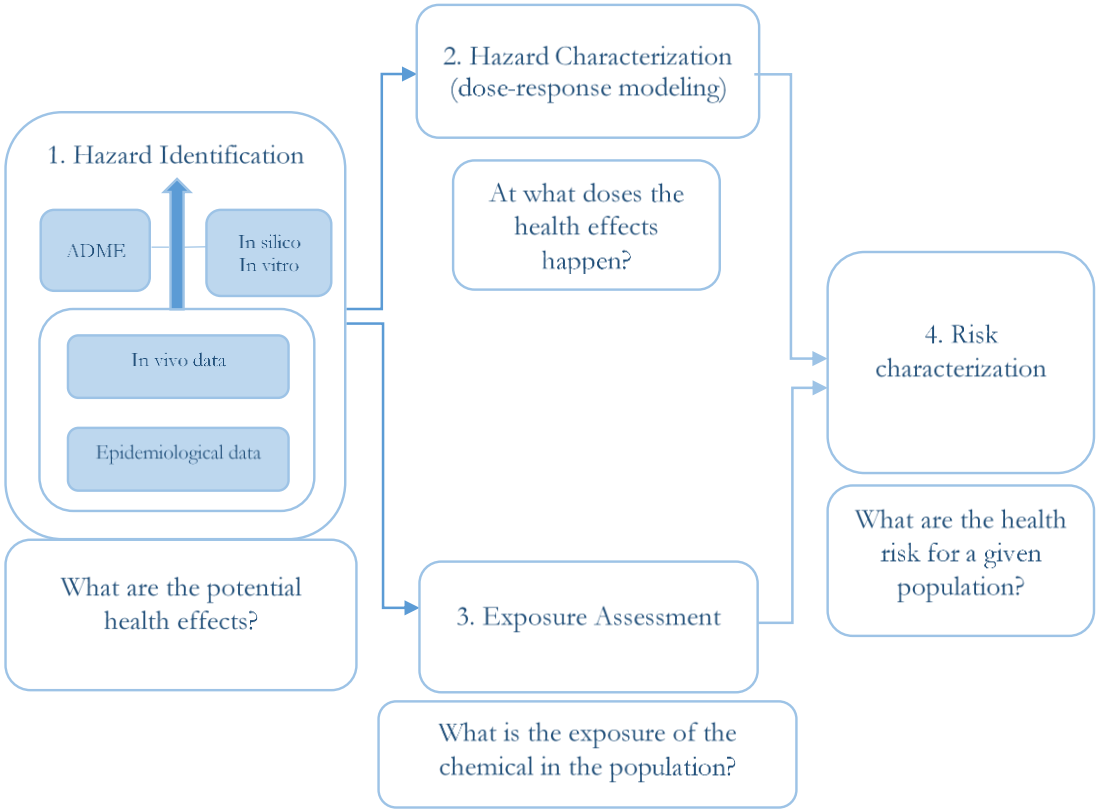


Figure 3. Classical Risk Assessment Framework

Chemical risk assessment is a multistep process involving 1) hazard identification, 2) hazard characterization, 3) exposure assessment and 4) risk characterization (Figure 3), as initially proposed by the US National Research Council (National Research Council Committee on the Institutional Means for Assessment of Risks to Public 1983). During the first steps, the evaluations are conducted to elucidate if the chemicals may cause a health effect to humans, and if such, at which dose that effect may happen in order to establish a safety level for regulatory purposes. Exposure assessment aims to determine at what levels of chemicals are exposed to, either using indirect methods like questionnaires or direct methods, with "*biomarkers of exposure*". Finally, the risk

characterization process integrates the levels of exposure with the hazard characterization information, to establish the probability that a given population may develop an adverse health effect due to such chemicals accounting for model uncertainties.

Major milestones in chemical risk assessment include the creation of the EPA in the US (1970) and their Toxic Substances Control Act (TSCA or TOSCA) in 1976. The list of regulated chemicals by TSCA includes all chemical substances manufactured or imported into the United States prior to December 1979, including some 8,800 chemicals imported or produced at quantities above 5 tons. At European level, the equivalent legislation is Registration, Evaluation and Authorization of Chemicals (REACH) adopted by the European Parliament and Council in 2007 to improve previous legislations in terms of human health and environment protection and to create the independent and mature regulatory agency ECHA. Before that time, in 2002 the European Commission also established the European Food Safety Authority (EFSA) that provides independent scientific advice and communicates on existing and emerging risks associated with the food chain. EFSA supports the European Commission, the European Parliament and EU member states in taking effective and timely risk management decisions that ensure the protection of the health of European consumers and the safety of the food and feed chain. EFSA also communicates to the public in an open and transparent way on all matters within its remit. In turn, at national level, France has been positioned in the spearhead of chemical risk assessment and food safety worldwide with the creation of The French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in 2010 to assess health risks in food, the environment and work, with the aim of enlightening public policy-making.

In 2019, world sales of chemicals were estimated at $4,363 billion, equivalent to the production of more than 2.3 billion tons of chemicals (excluding pharmaceuticals), which is approximately 300 kg per year for every man, woman, and child in the world. The contribution to the global gross domestic product is about 7% and the total number of jobs supported by all aspects of global chemistry industry was 120 million. Data from 22 chemical inventories from 19 countries and regions showed that over 350 000 chemicals and mixtures of chemicals have been registered for production and use, up to three times as many as previously estimated and with substantial differences across countries/regions (Wang et al. 2020). About 69000 are in commerce yet this figure may not exactly reflect the exact estimates for those chemicals registered before 2010 due to the lack of such information. A noteworthy finding is that the identities of many chemicals remain publicly unknown because they are claimed as confidential (over 50 000) or ambiguously described (up to 70 000). Nevertheless, during the last few years, an international transformative change has emerged to promote healthy, safety, security and sustainability industries, including the EU Green Deal among other policy strategies, intending to achieve standards of production, climate neutral, waste-free and non-toxic by 2050. The current EU chemical's legislative landscape targeting protection of human and environmental health comprises numerous legislations, each focusing on a specific domain or protection target comprising health, safety, security and sustainability summarized in Figure 4 (adapted from (Bruinen de Bruin et al. 2022)).

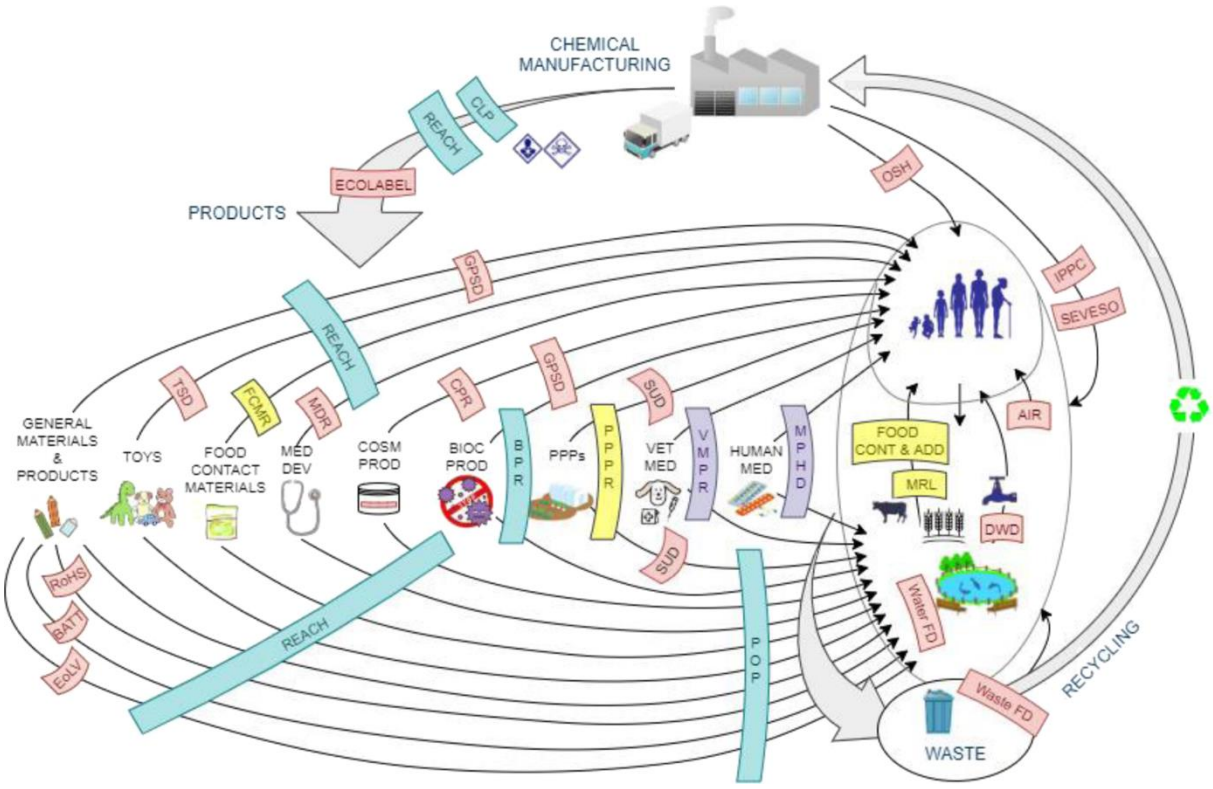


Figure 4. Legislation falling under the scientific remit of different EU institutions is labelled with different colors (blue: ECHA, yellow: EFSA, purple: EMA, red: EU Commission and Scientific Committees) (Bruinen de Bruin et al. 2022). CLP Classification, Labelling and Packaging of substances and mixtures, regulation (EC) No 1272/2008, REACH Registration, Evaluation, Authorisation and Restriction of Chemicals, regulation (EC) No 1907/2006, ECOLABEL EU Ecolabel, regulation (EC) No 66/2010, GPSD General Product Safety Directive 2001/95/EC, TDS Toy Safety Directive 2009/48/EC, FCMR Food Contact Materials, regulation (EC) No 1935/2004, MDR Medical Devices, regulation (EU) 2017/745, CPR Cosmetic Products, regulation (EC) No 1223/2009, BPR Biocidal Products, regulation (EU) No 528/2012, PPPR Plant Protection Products, regulation (EC) No 1107/2009, SUD Sustainable Use of Pesticides Directive 2009/128/EC, VMPR Veterinary Medicinal Products, regulation (EU) 2019/6, MPHD Medicinal Products for Human Use Directive 2001/83/EC, RoHS Restriction of Hazardous Substances in Electric and Electronic Equipment, Directive 2011/65/EU, BATT Battery Directive 2006/66/EC, EoLV End of Life Vehicles Directive 2000/53/EC, POP Persistent Organic Pollutants, regulation (EU) 2019/1021, Water FD Water Framework directive 2000/60/EC, Waste FD Waste Framework directive 2008/98/EC, DWD Drinking Water Directive 98/83/EC, MRL Maximum Residue Levels of Pesticides, regulation (EC) No 396/2005; FOOD CONT & ADD Food Contaminant regulations (EEC) No 315/93 and Food Additives regulation (EC) No 1333/2008, AIR Ambient Air Quality and Cleaner Air for Europe directive 2008/50/EC, SEVESO Seveso III directive 2012/18/EU, IPPC Integrated Pollution Prevention and Control directive 2010/75/EU, OSH Occupational Safety and Health Legislation, including directives 98/24/EC, 2004/37/EC, 92/85/EEC 94/33/EC.

REACH addresses chemicals used in industrial processes and intentional chemical mixtures and chemicals added to products in the European Union (Coria et al. 2022), using a novel landscape in chemical risk assessment process to accommodate the modern industrial needs and public health demands (summarized in Figure 5). The ECHA's Integrated Regulatory Strategy Annual Report 2019 provided a mapping of REACH database (data from 2018) showed that 2683 chemicals registered at high volume production (> 100 tons) where in an uncertain area, and over 1300 were identified of high priority to generate further data to allow decision making. Since 2008, 233 substances have been identified as substance of very high concern (SVHC), including the last 9 additions of January 17th 2023, and included in the Annex IV Candidate (Authorization program). The REACH Restriction program is not limited to SVHCs but also seeks to ensure that the risks from hazardous substances are properly controlled by prohibiting individual problematic uses of specific substances. The current number of listed SVHCs is much lower than the 1500 predicted by the agency in 2013, raising concern about the evaluation process. An economic analysis of main determinants showed that regulators were less likely to place a substance on the list if it is currently

being produced or imported in the European Economic Area (EEA) (Coria et al. 2022). Evaluations under REACH have been also discussed due to the use of non-standard approaches or lack of transparency (Borchert et al. 2022; Ingre-Khans et al. 2019).

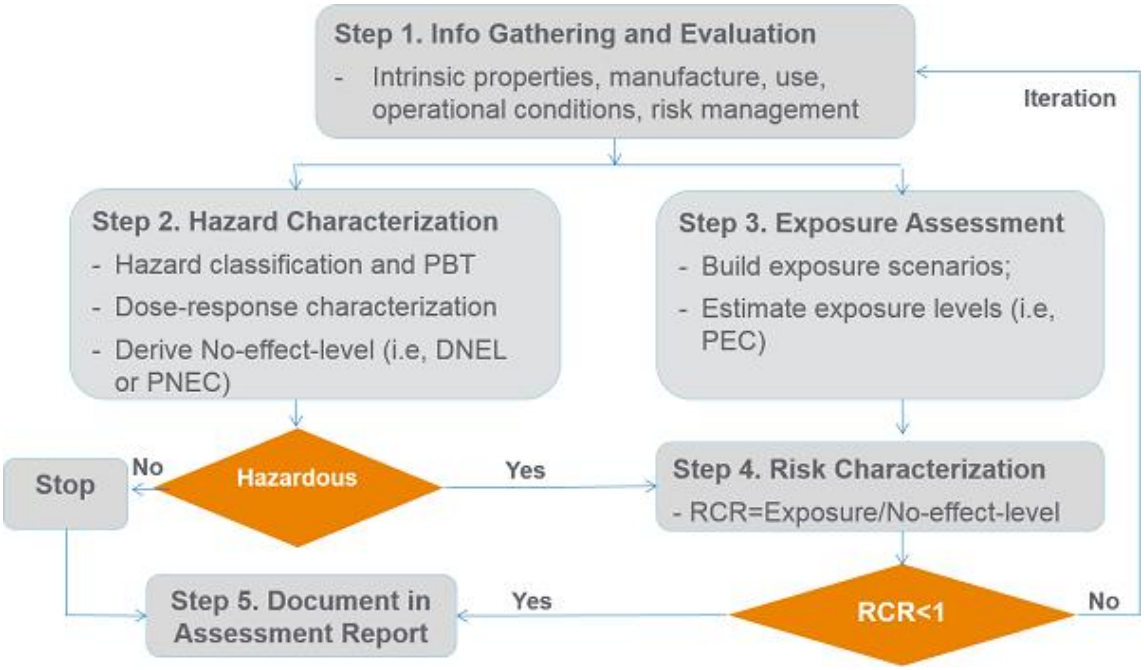


Figure 5. Risk Assessment Framework under REACH (Source ECHA)

1.3 Characterization of human chemical exposure

The major routes of human exposure to environmental chemicals are dermal, inhalation and oral, being the last one, the main route of exposure among general population for a vast majority of chemicals, and especially relevant for POPs. Modern synthetic chemicals, currently used for industrial and consumer applications are ubiquitous, which may be released to the indoor environment and accumulated in dust, thus their exposure pathways become more diversified. Exposure assessment of food contaminants can be conducted using 1) dietary modeling approaches (i.e. indirect methods) and 2) biomarker-based methods (i.e. direct methods). The dietary modeling approach implies the integration of chemical concentrations in a representative sample of food samples or their combination ("composite" samples) with the levels of consumption of those foods among the general population or specific subgroups. Different methods exist to conduct the integration, globally classified as determinist of probabilistic methods, yet mixed designs are often used. To compute deterministic (or direct) estimates, the summary contamination data and the consumption data are combined assuming independence between consumption (Cπ,j) and contamination (Tj) using the equation (1).

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \bar{C}_{\pi_0,j} \cdot \overline{T}_j \qquad (1)$$

where: $\hat{E}_{\pi_0}$ is the normalised exposure of sample population π_0; $\bar{C}_{\pi_0,j}$ is the arithmetic mean of normalised consumption of food *j* by population π_0 and $\overline{T}_j$ is the arithmetic mean of contamination of food *j*. Deterministic methods are reductionists of real-life complexity of variability and uncertainty of data distributions, thus conducted under multiple reductionist assumptions, for instance using single contamination point or uniform distribution of consumption.

Probabilistic approaches extend those estimates by considering the full distributions of consumption and/or contamination, allowing estimating the underlying variability and uncertainty, either using non-parametric approaches (without assumptions of distribution shapes) or parametric ones (characterizing the distribution parameters) (Figure 6).

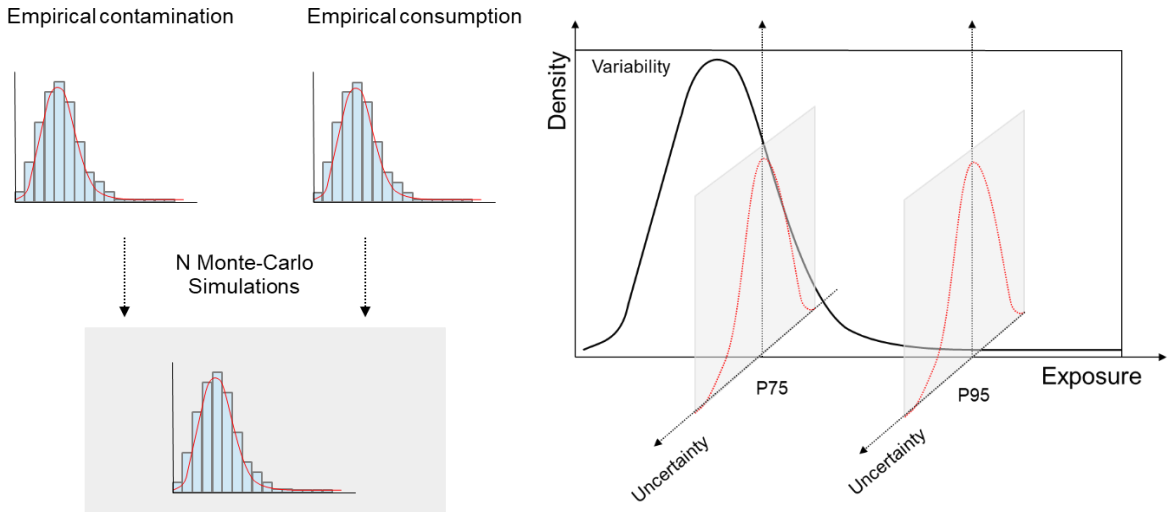


Figure 6. Quantitative exposure assessment framework (Cano-Sancho and Marin 2021)

Whereas probabilistic dietary exposure assessment methods have been mainly used for chemical risk characterization purposes (Cano-Sancho et al. 2011a; Cano-Sancho et al. 2012), poor attention has received from epidemiologist yet their potential to outperform traditional direct exposure estimates on reducing the exposure measurement error has been suggested (Nieuwenhuijsen et al. 2006).

Biomonitoring-based methods

The biomarkers has become the gold-standard approach in chemical exposure assessment due to its capacity to reflect the internal individual exposure, minimizing the large uncertainties (e.g. cooking processing or bioaccessibility) and accounting for inter-individual variability associated to ADME processes (Absorption, Distribution, Metabolism and Excretion) (Paustenbach and Galbraith 2006). Biomarkers are nowadays widely applied either for monitoring exposure levels among general population, but also in epidemiological studies has become the referent method. During the last decades the technological developments has allowed a fast evolution of mass-spectrometry based methods for biomarkers (Figure 7), increasing the sensitivity, selectivity and reproducibility of approaches with larger coverage of compounds and smaller volumes of samples. Since the first biomarker (lead) was used in the NHANES (1976-1980), the number of chemicals in the most comprehensive biomonitoring program has been growing each year until environ 400 chemicals covered nowadays. The pathways of method development have been paved by major milestones in chromatography and mass spectrometry, including the appearance of triple quadrupoles at end of the 1970s, and the emergence of high-resolution mass spectrometers (Time of Flight and Orbitrap) afterwards.

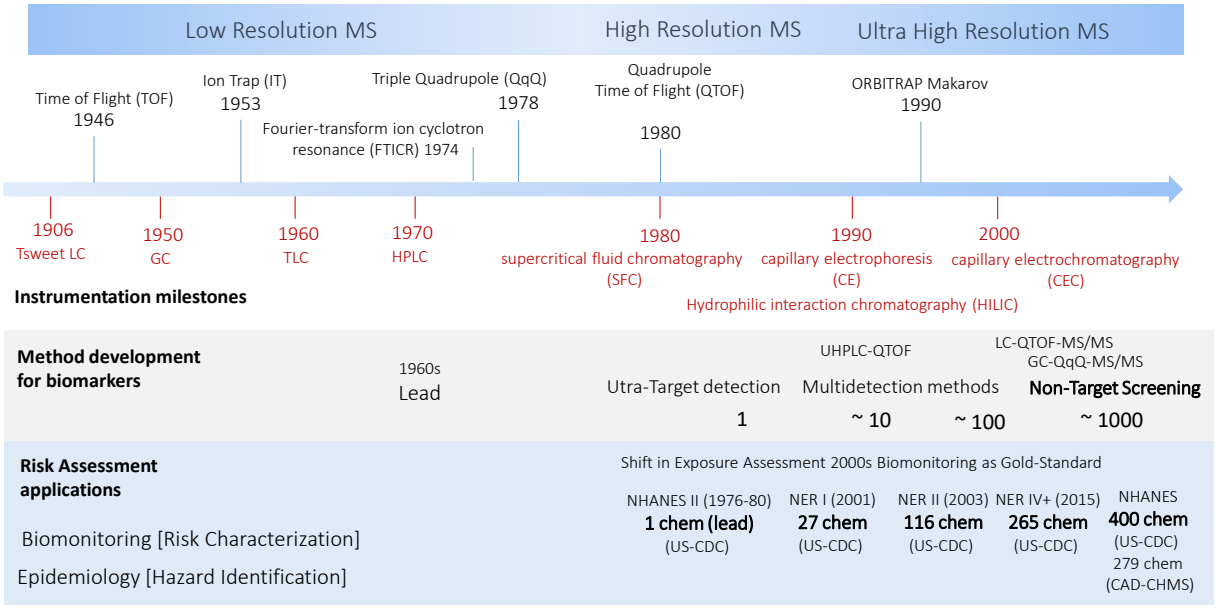


Figure 7. Time-line of major milestones on analytical development overlapped with biomonitoring advances

The design of analytical strategies for chemical biomarkers is multifaceted, requiring a multidisciplinary expert knowledge involving not only advanced competences on analytical instrumentation, but also physicochemistry (i.e. to develop efficient separative strategies with chromatography) or biology (i.e. to identify the best biological matrices of chemical by-products of phase-1 or -2 metabolism). Ultra-target and multi-targeted methods remain the referent method in chemical exposure assessment, intended either for risk characterization or epidemiological applications, ensuring the analytical performance with robust quality assurance/quality control (QA/QC) standards. Nonetheless, during the last decade, the raising of high-resolution mass spectrometry (HRMS) and bioinformatics tools has favored the development of non-targeted

methods, intending to provide a global chemical fingerprint instead of compound specific characterizations.

1.4 Life course exposure to environmental chemicals

1.4.1 Early-life human exposure to chemicals

From a life-course perspective, the first contact with chemicals happens *in utero*. Studies on human embryonic tissues showed for instance that PFAS can be found in fetal tissue during the first trimester (mean 9.5 weeks of gestation) suggesting that contaminated histotrophic secretions from endocrine organs may be the first exposure source (Mamsen et al. 2019). Around 12 weeks of gestation the maternal blood flow stars to nourish the fetus through the placenta becoming the main source of fetal exposure to chemicals because many of them can cross the placental barrier through simple diffusion, facilitated or mediated diffusion and endocytosis depending on their molecular weight (Mathiesen et al. 2021). The transplacental transfer of chemicals may differ among chemical families, for instance organochlorine pesticides showed larger transfer than PCB or PFAS (Björvang et al. 2021). Some studies have shown the capacity of placental enzymes of metabolizing the chemicals, yet little information is currently available for most chemicals. Sexually dimorphic accumulation of POPs have been observed in human fetuses suggesting than male fetuses accumulates more POPs than females, a trend also observed in the placental levels of POPs (Mathiesen et al. 2021).

Prenatal exposure to environmental pollutants, is thus determined by maternal lifestyle, environment and diet. Using a dietary modeling approach combining dietary intake patterns of pregnant women from the EDEN and ELFE cohorts and food contamination data of over 441 compounds from the ANSES database, six main mixture patterns were found among French women (Traoré et al. 2018). Among the most relevant mixtures during pregnancy common among cohorts, there is the TE-F-PAH: the substances common to each mixture were composed mostly of trace elements, five furans and the PAH pyrene. The "PCB-BFR-Aso-MeHg" mixture identified from EDEN before pregnancy was also identified from EDEN and ELFE during pregnancy, composed of composed of three non-dioxin like (NDL) PCBs, three dioxin like (DL) PCBs, seven BFRs and two trace elements. These results showed the complexity of distributional patterns of chemical compounds in mixtures based on women's dietary habits. (Traoré et al. 2018).

1.4.2 Early post-natal human chemical exposure: from breastfeeding to food diversification

Once the child is born and the umbilical cord is broken, the exposure pathways of chemicals extend to its extra-uterine environment and most importantly, its food, being milk (maternal or industrial) the first food system during the first years of development. During lactation a wide range of chemicals can be transferred to milk via passive diffusion, including more lipophilic chemicals such as POPs transported with lipids as a major energy source of infants (Landrigan et al. 2002). The International Agency for Research on Cancer (IARC) Exposome Database on environmental chemicals has registered over 180 unique congener from a variety of families including PCDD/Fs, PCBs, PBDEs, OCPs, PFAS or phthalates (Neveu et al. 2017). The lipid richness of breast milk favors the transfer of POPs that has shown average serum/milk partition ratios above or around the unit for most congeners. For instance for PCDDs that mean ratio range between 1.16 and 5.52. In the case of PBDEs the variability is even larger, form 0.69 (BDE47) to 24.95 (BDE209) (Mannetje et al. 2012).

The introduction of other foods than milk often begins between the 4th and 6th month, overlapping the recommended duration of breastfeeding, with exception of more allergenic foods like nuts commonly introduced after the 12th month (Banti et al. 2016). Introduction of novel foods also entails diversification of chemical exposures, hence another period that raise specific concerns for several food contaminants. For instance, in recent studies we have shown that the early

introduction of bread, results on a substantial increase of exposure of mycotoxins deoxynivalenol and enniatin B1, being diversification period the main age-related risk group (Gallardo et al. 2022). The dietary diversification continues until the establishment of the dietary pattern, that often happen before the 3 years, when the diversity reach a plateau, mirroring in many cases, the parents dietary patterns (Chong 2022).

1.5 Linking chemical exposome and health: from cell to populations

As introduced in the general chemical risk assessment framework (Section 2), hazard identification is "the process of determining whether exposure to an agent can cause an increase in the incidence of a health condition, characterizing the nature and strength of the evidence of causation" (Chiu and Rusyn 2018). This component of risk assessment is commonly accomplished by the integration of multiple streams of evidence, namely human epidemiological, animal in vivo, in vitro (mechanistic) and/or in silico, either for regulatory decision-making or state-of-the-science purposes (Whaley et al. 2016). This step is a prerequisite to conduct dose-response modelling and estimations of safety thresholds (points of departure) to be further applied in risk characterization. Integration of scientific evidence is commonly achieved by "weight-of-evidence" frameworks intending to gather, weighting and evaluating the evidence from multiple sources to support evidence-based conclusions (OCDE 2019).

The main reason of such multi-faceted process arise on the fact that causal relationships between chemical exposures and human health cannot be directly proved by ethical reasons preventing human experimentation at controlled doses of environmental contaminants. Hence, toxicological evaluations in animal or cell-based models are required to infer causality on humans based on structured causal chain of events or modes of action, plausibly linking the chemical exposures and health disorders at individual or population level. The adverse outcome pathway (AOP) framework has been proposed to simplify the complex biological mechanisms underlying those causal chains of events to support regulatory decision-making and embraced by international organizations (Villeneuve et al. 2014). The AOP is a structuration of the causal sequence of events, from first molecular triggers resulting of direct chemical stressor binding or interaction (i.e. known as molecular initiating events ,MIE) towards a disease manifested at population level (i.e. adverse outcome, AO), through different anchored molecular processes (Key events, KE) happening at cellular or organ level (Figure 8).

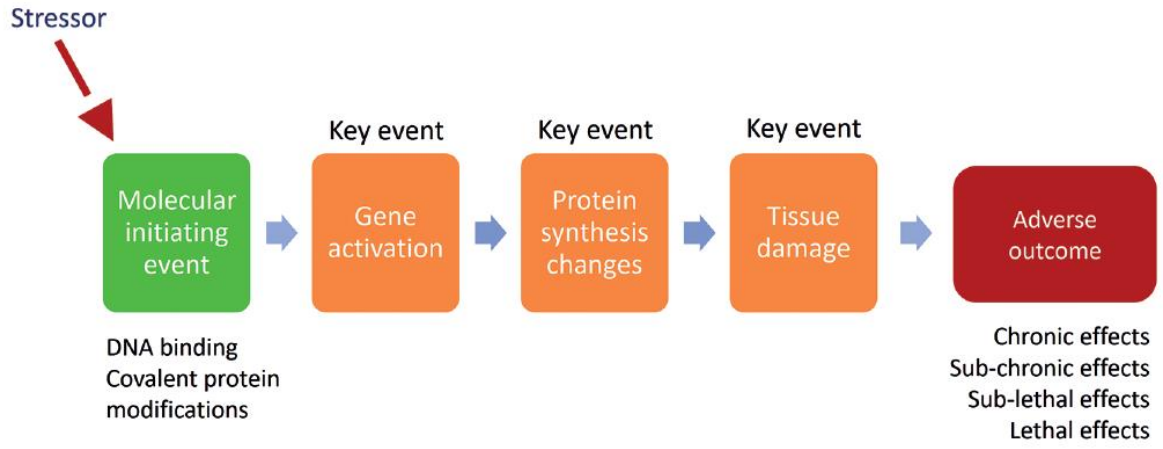


Figure 8. Adverse outcome pathway framework example

Chemical risk assessment has traditionally relied on animal evaluations of apical endpoints, yet 21st Century Toxicity Testing vison provides a clear shift towards the "3R" principles of Replacement,

Reduction and Refinement in order to fully commit to animal welfare. On this regard, New approach methodologies (NAMs) based on *in-silico* predictive toxicology and in vitro methods have been extensively developed in order to accommodate the novel paradigm in chemical risk assessment in term of the growing number of chemicals in the market, but also the clear ethical aspects that confront nowadays the conventional use of a large number of animals. The development of high-throughput screening (HTS) based on metabolomics (i.e. comprehensive characterization of metabolites and lipids) as NAM in chemical risk assessment is one of the examples of recent developments in toxicology using omic technologies that can support the identification of novel hazards and associated mechanisms to inform modes of action (MoA) from in vitro studies (Malinowska et al. 2022).

Environmental epidemiological studies have substantially contributed to inform chemical risk assessment, however those are often not specifically developed for such purpose, hence a dual vision of environmental epidemiological data exist between risk assessors and epidemiologists demanding further collaboration of bridge the gaps (Déglin et al. 2021). The development of NAMs has also being pointing out towards novel epidemiological study designs, not only to generate hypothesis, but also to decipher potential MoA by integrating novel molecular/omic technologies (Lanzoni et al. 2019). Actually, the integration of effect biomarkers (biological markers of early or preclinical effect) in epidemiology has shown proved potential to gain insight on the mechanisms between environmental exposures and health, yet the systematic application remains limited (Zare Jeddi et al. 2021).

1.6 The role of environmental epidemiology to characterize the associations between chemical mixtures and health

The complex exposure patterns of chemical mixtures have been illustrated in Section across different age-stages and biological matrices, hence a natural subsequent question could be: what is the joint association of those chemicals with health disorders? In epidemiological studies, three main purposes arise from mixture evaluations: a) identifying the individual associations accounting for the co-exposure with other chemicals in a mixture, b) identifying interactions between chemicals and c) characterizing the global effect of the mixture (Braun et al. 2016). A list of statistical challenges underlie the motivating question (Lazarevic et al. 2019):

- High correlation between exposures can lead to inflated standard errors and instability in effect estimates
- Differing degrees of measurement error among exposures can bias effect estimates
- Insufficient power to estimate small effects in the presence of measurement error, multicollinearity, small sample size, interactions, and nonlinearity.

The number of statistical methods and computational software developed to overcome those challenges to answer complex question related to mixture effects have been growing during the last decade, since first publications on multipollutant issues were appeared during early 2010s (Joubert et al. 2022). A summary of methods is provided in Figure 9 published by (Joubert et al. 2022). Whereas development and application of mixtures methods have been mainly motivated by the joint effects and interactions between chemical pollutants, little attention has been paid to chemical mixtures of toxicant chemicals and nutrients that may present a counterbalancing effect.

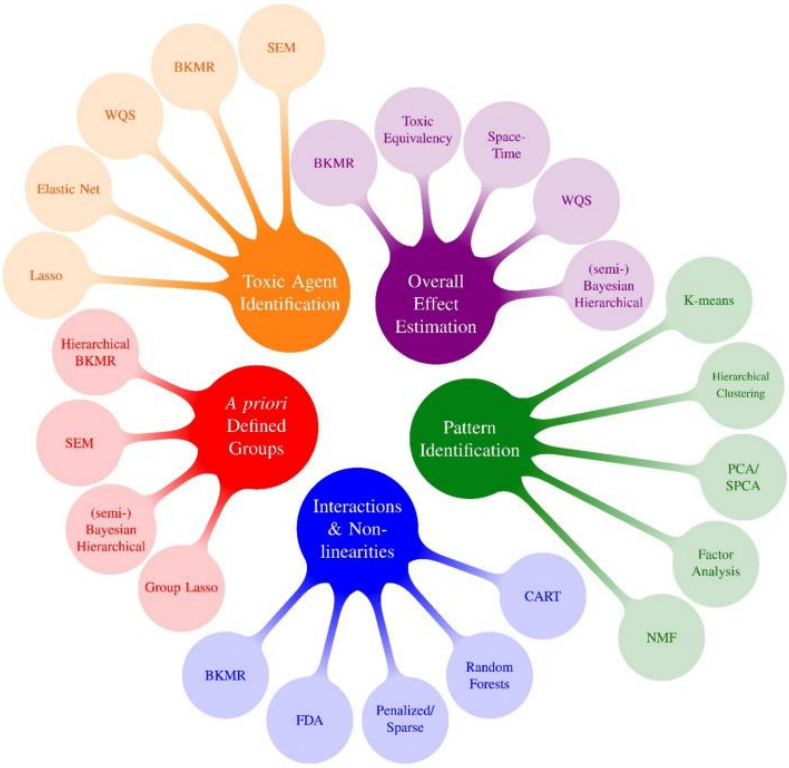


Figure 9 Mixtures Methods x Research Questions 1: Methods Preceding PRIME. 1 Research questions following Gibson et al., 2019 review: (1) Overall effect estimation: What is the overall effect of the mixture and what is the magnitude of association? (2) Toxic agent identification: Which congeners or chemicals are associated with the outcome? What congeners/chemicals are most important? (3) Pattern identification: Are there specific exposure patterns in the data? (4) A priori defined groups: What are the associations between an outcome and a priori defined groups of exposures? (5) Interactions and non-linearities: Are there interactions between exposures? Is the exposure-response surface non-linear? (6) Exposure-response relationship: What is the exposure-response relationship between each chemical and the outcome? Because almost all methods that investigate interactions also characterize potentially nonlinear exposure-response functions, we group questions #5 and #6 into a single bubble in this figure (Joubert et al. 2022).

1.7 Molecular epidemiology to decipher mechanisms and supporting biological plausibility

Molecular epidemiology was born in the 1980s as a novel approach to gain insight on the causal relationships in observational studies incorporating biomarkers to reveal mechanisms and events occurring along the theoretical continuum between exposure and disease (Perera and Weinstein 1982). Biomarkers of effect, represent organism's molecules whose concentration levels are altered as an internal response to a health disorder or disease, either metabolites, lipids, proteins or epigenetic markers among others (NRC 2006). Interestingly, biomarkers of effect can help in identifying early effects in humans at low doses, establish dose–response relationships, explore mechanisms and increase the biological plausibility of epidemiological associations. In addition, effect biomarkers can improve the risk assessment of specific chemical families as well as exposure to chemical mixtures (Zare Jeddi et al. 2021).An illustrative example of biomarker of early effects is the ratio of sphingolipid metabolites, sphinganine and sphingosine (Sa:So) due to the alteration of the enzyme ceramide synthase triggered by the myocotoxin Fumonisin B1 (FB1) (Merrill et al. 2001). FB1 has a sphingoid backbone, and the potential for ceramide synthase inhibition by FB1 was recognized and then rapidly demonstrated in experimental animal models. Inhibition was demonstrated as a modulation of two physiologically important precursors in sphingolipid production, sphinganine (Sa) and sphingosine (So), leading to an increase in the ratio of Sa/So. This mechanism appears to be on the causal pathway for toxicity of FB1, depicted in Figure 10 (Turner and Snyder 2021).

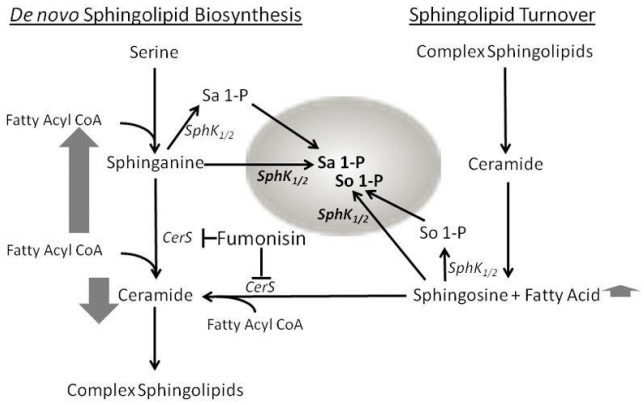


Figure 10. Enzymology of FB-induced sphingoid base 1-phosphate accumulation in RBC. RBC cannot synthesize sphingolipids de novo and lack the ability to de-phosphorylate sphingoid base 1-phosphates. However, RBC have the ability to phosphorylate sphingoid bases via sphingosine kinases (SphK1/2). Thus, the sphingosine (So) and sphinganine (Sa) that is phosphorylated in the RBC is produced outside the RBC. In animals large amounts of So and Sa are produced in liver, kidney, and other tissues following FB inhibition of ceramide synthase (CerS). The So and Sa that is produced in tissues following FB exposure is a likely source of substrates for the SphKs in the RBC following FB intake, however, RBC can also take up and store So 1-P and Sa 1-P from endothelial cells. The vertical arrows next to Sa and So represent the relative magnitude of the accumulation when CerS is inhibited. The downward arrow next to ceramide indicates the decrease in ceramide biosynthesis in tissues (Riley et al. 2015).

Whereas this attractive biomarker was validated in animal studies, the validation in human studies with lower exposure levels to FB1 has become more complex. As part of my PhD I conducted a controlled time-course trial in order to gain insight on the timing between the exposure to fumonisins after the corn-based food intake. The results showed that the ratio reached a peak at the fourth day after a FB dietary intake and then declined (Figure 11), suggesting that enzymatic alterations were reversed in few days at low concentrations (Cano-Sancho et al. 2011b). A more recent and large-scale human study conducted in Guatemala with 1129 paired estimated FB intake and urinary samples, provided a first robust evidence in humans of the associations between FB and the disruption of sphingolipid biosynthesis. In this case, instead of measuring the parent sphingolipids, the phosphate adducts (Sa-Pi/So-Pi) were measured and compared to urinary FB1 measures obtaining strong correlations ($p < 0.0001$, $r = 0.49$) (Riley et al. 2015). In applied studies, the ratio Sa:So has been used either as marker of exposure and marker of early risk of esophagus cancer.

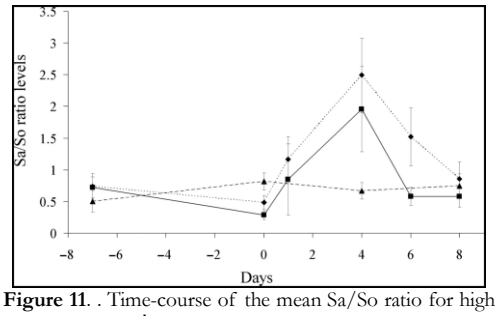


Figure 11. . Time-course of the mean Sa/So ratio for high exposed (H,), low exposed (L,) and non-exposed (C,).

Analytical challenges

The illustrative example of the biomarker Sa:So was discovered driven by biological hypothesis developed on the basis of extensive toxicological knowledge of Fumonisins developed during decades. Such approach may be prohibitive nowadays due to large economical cost and ethical implications of animal experimentation, hence alternative data-driven methods supported by high-throughput omic technologies have been proposed to contribute such research. One of those omic technologies is metabolomics, that entails large-scale systematic identification and further quantification of metabolites from biological organisms at any moment (Fiehn 2002). These small endogenous metabolites are metabolic products that play essential roles in biochemical pathways, which may be disrupted and linked to disease-state disorders under pathological or stress conditions. Main technologies for metabolomics profiling include nuclear magnetic resonance (NMR) and HRMS, which may be used to develop analytical strategies toward targeted compounds or as introduced above, more global metabolic fingerprinting with non-targeted methods followed by bioinformatics pipelines to support the annotations and semi-quantifications. The application of metabolomics profiling in environmental epidemiological studies have been steadily growing during the last few years embraced by the exposome concept raising, showing the discovery potential in different case-studies (Guo et al. 2022).

Statistical challenges

Integration of effect biomarkers data in environmental epidemiology has been growing during the last 5 decades since first proposed in the 1980s as alternative to toxicology to gain insight on the mechanisms in epidemiological studies (Links et al. 1995; Perera and Weinstein 1982). In the case study of carcinogenic agents related to leukaemia, it was proposed that if preclinical biomarkers would be also related to environmental exposures, a causal link could be supportive, raising the "meet-in-the-middle" (MITM) concept (Vineis and Perera 2007). During the last few years high-throughput technologies has favoured scaling up the data generation of endogenous biomarkers, raising novel statistical challenges to integrate and analyse them in epidemiological settings. On this regard, the MITM concept has evolved to accommodate omic scale data and overcome the associated challenges. A first proposal focused on metabolomics data integration, conceived a sequential strategy to first identify exposure biomarkers [E→Y] and metabolomic biomarkers [M→Y], and second, the intersecting intermediate biomarkers as potential causal markers [E→M] (Chadeau-Hyam et al. 2011). Traditionally, casual mediation analysis with a single mediator [M] or intermediate variable, encompasses statistical methods to decompose the total effect between an exposure [E] and an outcome [Y] into their direct effects and those indirect effects, mediated by M. This statistical framework fits the continuum between environmental exposures and related biological fingerprints (e.g. exposure and effect biomarkers) along the adverse outcome pathways during the disease progression (Figure 12 Panel A) with a different potential structures of associations as depicted in Figure 12 panel B to E [adapted from (Cadiou et al. 2021)]. For instance, the association between E and Y can be partially mediated by M (Panel B), fully mediated by M (Panel C), not mediated at all by M but also explained by it (Panel D) or even, some cases where M may be a consequence of an outcome Y causally related to E (Panel E). These different causal scenarios may become highly complex among those diseases related to multiple exposures and/or when chains of multiple mediators may be biologically connected (Daniel et al. 2015; Taguri et al. 2018), that is the case of high-throughput profiling platforms (Vineis et al. 2013).

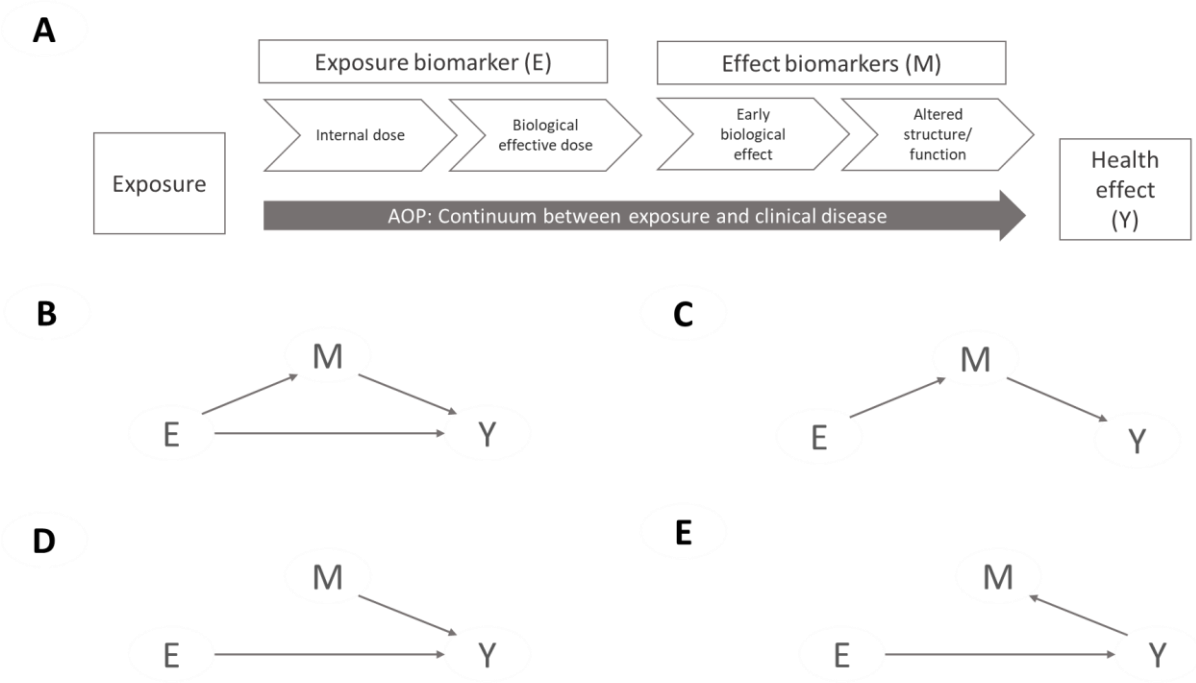


Figure 12. Causal mediation structures linking exposures (E), mediators or intermediary variables (M) and outcomes (Y)

In a recent study, we critically review the current state-of-the-science about statistical approaches used to integrate endogenous biomarkers in environmental-health studies linking chemical exposures with health outcomes (Babin et al 2023, Submitted). Multivariate regression models were the most predominant statistical method reported in the published workflows, however some studies applied latent based methods or multipollutant models to overcome the specific constraints of omic or exposure of data. A minority of studies used formal mediation analysis to characterize the indirect effects mediated by the endogenous biomarkers.

2. SCIENTIFIC CONTRIBUTIONS

This section summarizes the main results of recent researches (period 2017-2023) where I have contributed to gain insight on the synthetic chemical exposome and its relationships with health. This period reflects the results since the end of my second postdoctoral visit and the first period at my current hosting unit LABERCA (since October 2016). This section does not follow a chronological ordering; instead, the contributions have been structured in main critical windows of chemical exposure during the lifespan with a focus on female health (Summary in Figure 13). Hence, a first chapter is focused on the perinatal exposure to environmental chemicals and its impacts on overweight/obesity programming, including cell-based studies conducted during my postdoc and two exposome-scale epidemiological approaches (NUTPOP and LACTACOL/LACTOMICS projects). A second chapter elaborates the research on internal chemical exposure of women at reproductive age and its impacts on reproductive disorders, such as endometriosis, ovarian function and in vitro fertilization (IVF) outcomes within the framework of the EndoxOMICS-β project. The final chapter shows the results of research about the chemical exposure of elderly women and its associations with the risk of breast cancer presence and aggressiveness from the collaborative ED-Cancer and METAPOP projects. In addition, some methodological axis have been developed to overcome some ongoing challenges on the application and analysis of biomarkers in exposome-health research (CREATIVE and MUTLIBLOC projects)

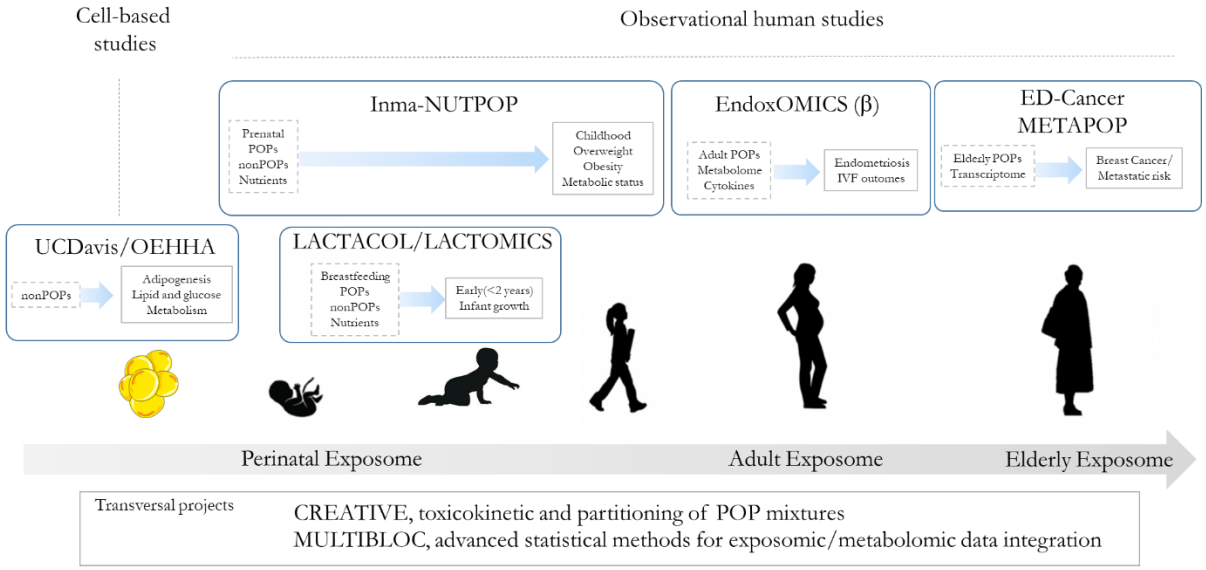


Figure 13. Summary of main research contributions from the last period (2016-2023).

2.1 Pre-natal exposure to environmental chemicals and childhood obesity

2.1.1 A cell-based approach to identify environmental metabolic disruptors

Metabolic disrupting chemicals (MDCs) are a class of compounds that alters susceptibility to metabolic disorders including obesity, type 2 diabetes mellitus or nonalcoholic fatty liver disease (NAFLD) (Heindel et al. 2015; Heindel et al. 2017). The obesogenic hypothesis was proposed by Blumberg and Grün in 2006, suggesting that some chemicals could contribute influence adipogenesis and contribute to the obesity pandemics (Grün and Blumberg 2006). Despite the terminology is quite recent, the metabolic disrupting effects of environmental chemicals have been shown for decades. For instance, we showed in a systematic review with meta-analysis to assess the links between the legacy chemical DDT with obesity in epidemiological and experimental studies (Cano-Sancho et al. 2017a), that altered liver and/or blood lipids in rodents were already documented in the 1970s (Darsie et al. 1976) and 1980s (Appleton et al. 1981). The mechanisms by which MDCs contribute to obesity and metabolic disorders are not fully elucidated, however current research has shown their ability to alter 1) energy intake by modulating neuronal behaviour responsible for food intake; 2) epithelial transport in the intestine and the microbiota composition; 3) biosynthesis and probably also the release of gut peptides; 4) insulin release from β cells or 5) energy storage by targeting the brain, skeletal muscle, white adipose tissue and the liver (Nadal et al. 2017).

In vitro adipocyte models are useful tools to screen the potential obesogenic effects of environmental chemicals due to their unique features to study major energy regulation functions such as lipogenesis, lipolysis or glucose uptake, in addition of morphological changes directly related with obesity outcomes such as adipogenesis or hypertrophy (Hammarstedt et al. 2018). One of my postdoctoral researches was focused on developing a battery of test to evaluate the obesogenic potential of environmental compounds, a collaborative project between University of California at Davis and the Office of Environmental Health Hazard Assessment (OEHHA), a Californian Branch of the US EPA. We used 3T3-L1 pre-adipocytes to test the influence of chemicals on the differentiation process or mature adipocytes to evaluate the impacts on lipolysis, glucose uptake or the alterations of related genes and proteins using quantitative PCR or Western-blots, respectively. We screened over 30 chemicals from National Toxicology Program on the glucose uptake assay, and selected four of them for a more comprehensive evaluation, considered chemicals of emerging concern: triphenyl and diphenyl phosphate (TPhP and DPhP); methyl and propyl parabens (MeP and PP).

TPhP increased pre-adipocyte proliferation and subsequent adipogenic differentiation of 3T3-L1 cells, coinciding with increased transcription in the CEBP and PPARG pathway (Figure 14). Treatment of mature adipocytes with TPhP increased the basal- and insulin stimulated- uptake of the glucose analog 2-[N (−7-nitrobenz-2-oxa1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG). This effect was ablated by inhibition of PI3K, a member of the insulin signaling pathway. DPhP had no significant effect on cell proliferation and, compared to TPhP, a weaker effect on adipogenic differentiation and on 2-NBDG uptake. Both TPhP and DPhT significantly enhanced the isoproterenol-induced lipolysis, most likely by increasing the expression of lipolytic genes during and after differentiation (Cano-Sancho et al. 2017b). Methyl- and propyl- parabens increased adipogenesis of 3T3-L1 white adipocytes but not brown adipocytes. In white adipocytes, methyl paraben increased glucose uptake and both parabens reduced basal lipolysis (Elmore et al. 2020).

These studies indicate that methyl- and propyl- parabens target the differentiation and metabolic processes of multiple types of adipocytes in a cell autonomous manner, and TPhP increases

adipogenic differentiation, glucose uptake, and lipolysis in 3T3-L1 cells through endocrine and noradrenergic mechanisms.

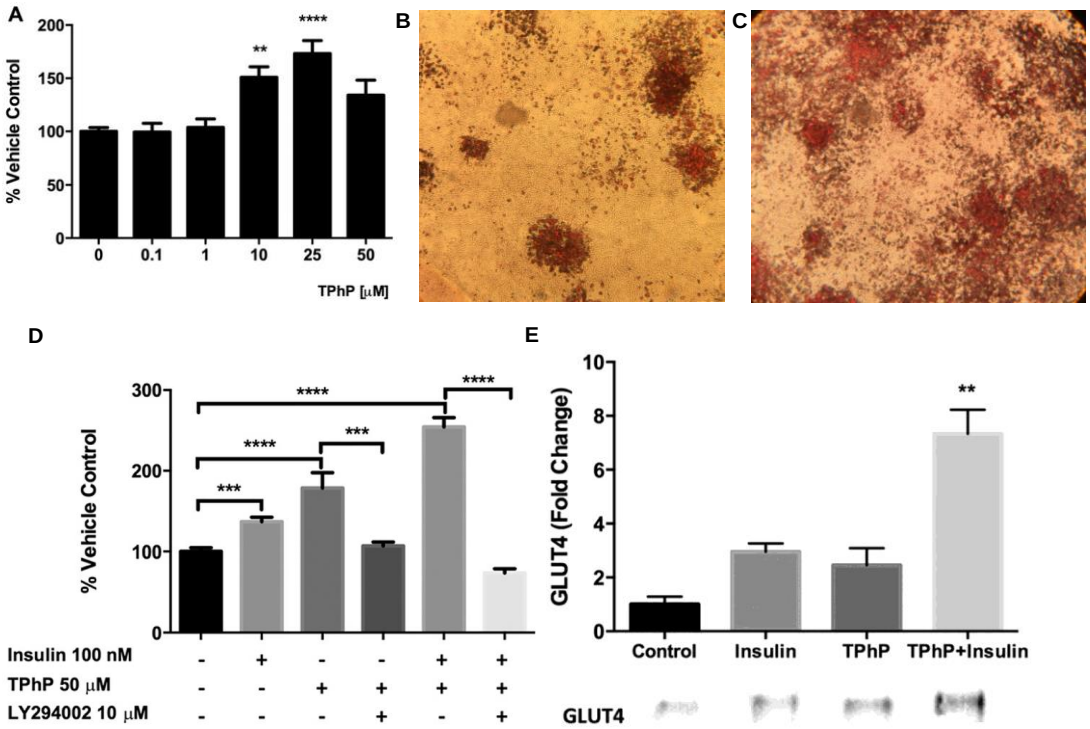


Figure 14. Panel A) Effect of TPhP on adipogenic differentiation of pre-adipocytes 3T3-L1 measured by staining neutral lipids using the oil red O assay. TPhP and DPhP were added to the weak differentiation cocktail during the whole differentiation process. Statistical analysis was performed using one-way ANOVA followed by a Dunnett's test to compare the different dose levels of TPhP and DPhP with the respective vehicle control. Results are represented by the mean and the standard error of the mean from three experimental replicates (n = 3). Significance levels are represented with asterisk as following: (*) P < 0.05, (**) P < 0.01, (***) P < 0.001. Morphological observation and Oil Red O staining of 3T3-L1 cells photographed using a microscope (X200) without treatment (Panel B) and treatment with TPhP 10µM (Panel C). Panel D) Inhibitory effect of LY294002 on 2-NBDG uptake stimulated by TPhP. Statistical analysis was performed using one-way ANOVA followed by a Dunnett's test to compare the different dose levels of TPhP with the respective vehicle control. Results are represented by the mean and the standard error of the mean from three experimental replicates (n = 3). Pinteraction = 0.005 for TPhP and insulin. Significance levels are represented with asterisk as following: (**) P < 0.01, (***) P < 0.001, (****) P < 0.0001. Panel E) GLUT4 translocation in 3T3-L1 cells exposed to vehicle control (DMSO 0.1%), insulin 100 nM, TPhP 50 µM or simultaneous exposure to insulin and TPhP for 15 min. The amounts of GLUT4 are expressed as values relative to those in cells treated with vehicle control. Statistical analysis was performed using two-way ANOVA followed by a Dunnett's test to compare the different dose levels of TPhP with the respective vehicle control. Results represent means ± SEM, n ± 2 independent experiments. Pinteraction = 0.07. Significance levels are represented with asterisk as following: (**) $P <$ 0.01.

2.1.2 Exploring the pollutant-nutrient interface during early pregnancy and their impact on child growth and metabolic status: results from the INMA birth cohort

Dietary intake is a major entry pathway for many chemicals in the body, either essential nutrients or unwanted contaminants, which may both be present in the diet and which may also share systemic mechanisms of transport or simultaneously act on essential signaling pathways required to maintain the biological systems of our organism. Conceptually, the interactions between nutrients and pollutants could be explained by a combination of coexposure and physicochemical commonalities with similar biological activity. The first two levels of confluence would be (1) due to shared exposure or dietary intake patterns (ie, coexposure) or (2) common mechanisms of absorption, transport, storage or excretion (ie, toxicokinetics). The third level of confluence relates to the biological interaction itself due to sharing similar molecular signaling pathways (ie, toxicodynamics). In a recent essay we have discussed the special case of lipophilic nutrients and POPs, which highlight the specific case where confluence may likely take at all abovementioned levels thereby increasing the probability of modification of final health effects (Cano-Sancho and Casas 2021).

In order to gain insight on such complex nutrient-pollutant associations we have conducted a study with the main objective of characterizing the joint associations and interactions between prenatal levels of POPs and nutrients on childhood obesity (Cano-Sancho et al. 2023). We used data from to the Spanish INMA birth cohort, on POPs and nutritional biomarkers measured in maternal blood collected at first trimester of pregnancy and child anthropometric measurements at 7 years. Six organochlorine compounds (DDE, HCB, β-hexachlorocyclohexane [β-HCH] and polychlorinated biphenyls 138, 153, 180) and four per-/PFAS were measured. Nutrients included vitamins (D, B12 and folate), polyunsaturated fatty acids (PUFAs), and dietary carotenoids. Two POPs-nutrients mixtures datasets were established: (i) OCs, PFAS, vitamins, and carotenoids (n=660) and (ii) OCs, PUFAs, and vitamins (n=558). Joint associations of mixtures on obesity were characterized using Bayesian Kernel Machine Regression (BKMR). Relative importance of biomarkers and 2-way interactions were identified using Gradient Boosting Machine, hierarchical group-lasso regularization, and BKMR. Interactions were further characterized using multivariate regression models in the multiplicative and additive scale.

The results showed the widespread presence of those POP chemicals during the first trimester, detected in all pregnant women supporting the early exposure of fetuses. The correlation analysis showed high positive correlations ($\rho > 0.5$) between POPs within families (Figure 15A and B), and mild or no correlation between families. In turn, p,p'-DDE was not correlated with PFAS and moderately correlated (ρ: 0.3-0.2) with PCBs, HCB, and β-HCH. Regarding the fatty acids, arachidonic acid (AA) and docosahexaenoic acid (DHA), both showed positive correlations with PCBs and negative correlation with β-HCH (ρ: 0.3 and -0.1, respectively). Similar but weaker association profiles were shown for eicosapentaenoic acid (Figure 15B), and LA was negatively correlated with PCBs and the rest of PUFAs. Most OCs and PFAS congeners were not correlated with dietary carotenoids and vitamins with few exceptions. For instance PFHxS showed mild positive correlations with most nutrients (ρ: 0.1-0.2), whereas p,p'-DDE was negatively associated with retinol (ρ: -0.3) (Figure 15A).

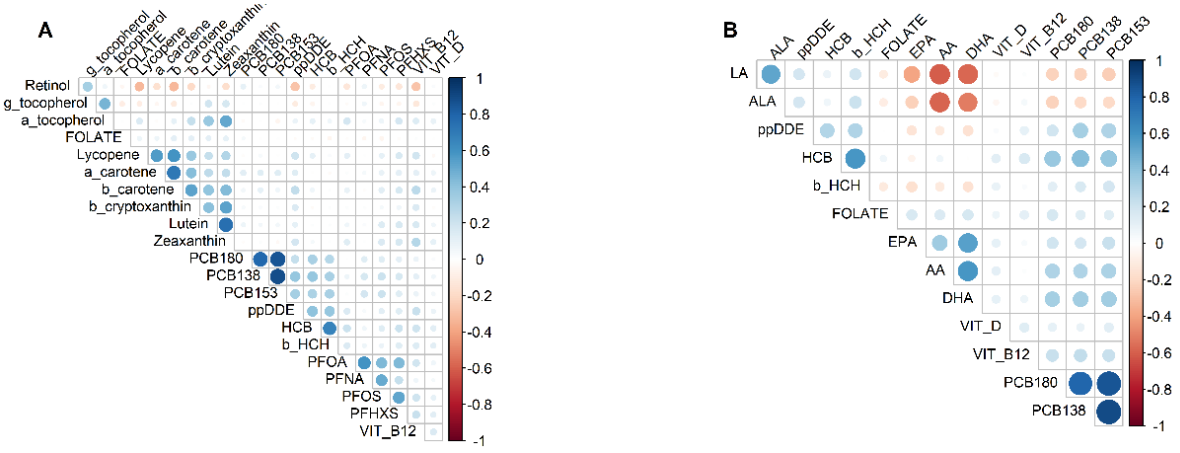


Figure 15. Spearman's correlation plots depicting the association strength between POPs and nutrients in the ANTIOX (Panel B, n=660) and PUFA (Panel A, n=558) datasets.

We observed a positive overall joint association of both POPs-nutrients mixtures on overweight/obesity risk, being HCB and vitamin B12 the biomarkers contributing the most. Recurrent interactions were found between HCB and vitamin B12 across screening models. Relative risk and 95% confidence interval (95% CI) for a natural log increase of HCB was 1.31 (1.11-1.54, $p_{interaction}$=0.02) in the tertile 2 of vitamin B12 and in the additive scale a Relative Excess Risk due to Interaction of 0.11 (95% CI, 0.02-0.20) was found (Figure 16A). Interaction between perfluorooctane sulfonate (PFOS) and β-cryptoxanthin suggested a protective effect of the antioxidant on overweight/obesity risk (Figure 16B). These results support that maternal nutritional status may modulate the effect of prenatal exposure to POPs on childhood overweight/obesity. These findings may help to develop biological hypothesis for future toxicological studies and to better interpret inconsistent findings in epidemiological studies.

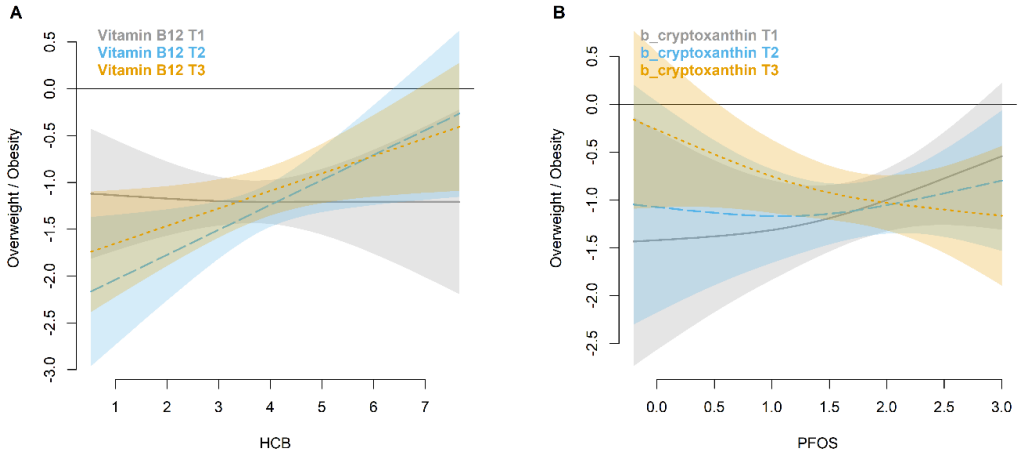


Figure 16. Interactions plots of associations between HCB and PFOS with overweight/obesity risk by tertiles of vitamin B12 (A) and b-cryptoxanthin (B), respectively

2.1.3 Infant exposure to environmental chemicals through breastfeeding and growth

We have conducted a comprehensive and integrative characterization of breast milk exposome and metabolome from women recruited within the Lactacol Cohort, presenting a pre-term delivery (<37 weeks). The analytical workflow was developed to conduct a comprehensive profiling of 102 POPs and dietary nutrients and 784 endogenous metabolites combining ultra-trace targeted methods and non-targeted methods. The results showed the widespread presence of most analyzed POPs, and in general exceeding those maximum levels allowed by the European regulation for raw milk and dairy products intended for human consumption (Figure 17). The correlation analysis of targeted lipophilic chemicals in breastmilk revealed strong positive associations between ω3 PUFAs (docohexaenoic acic or DHA, docosapentaenoic acid or DPA and eicosapentaenoic acid or EPA) and most POPs, most substantially for TCDD, non-coplanar and non-dioxin like PCBs (Figure 18). These toxicants were also strongly correlated to saturated FAs such as pentadecanoic acid (15:0), palmitic acid (16:0), stearic acid (18:0) and arachidic acid (20:0). Some strong inverse correlations were also noticed for ω6 PUFAs and non-coplanar PCBs, and specially docosapentaenoic acid (22:5n-6), docosadienoic acid (22:2n-6) and linoleic acid (18:2n-6) exhibiting the strongest negative correlation with most POPs. The bivariate correlation analysis was extended with sparse Partial Least Squares allowing the efficient integration of two correlated matrices and the identification of the most relevant variables and their networks, displayed in Fig. 4. The relevance networks display the topological associations between FAs and POPs considering a cut-off level of bivariate correlation of 0.34 to favor the visualization of most relevant associations. This approach showed that FAs, 15:0 (pentadecanoic acid) and 20:1 (eicosenoic acid) appeared as central nodes on the association network with PBDEs, highlighting the strong negative associations between 15:0 and PBDE99 and positive associations between 20:1 and PBDE99 or the sum of PBDEs. Another network was built around total lipids and energy, negatively associated to PCBs and around the saturated FAs 20.0 (arachidic acid) and 18.0 (stearic acid) displaying positive associations with coplanar PCBs such as PCB114.

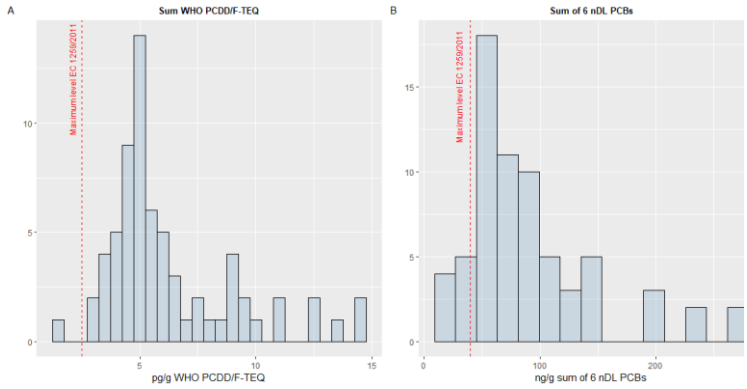


Figure 17. Histograms depicting the levels of sum WHO PCDD/Fs TEQ (A) and sum of 6 non-dioxin like PCBs (B) in human milk with the respective maximum levels established by the COMMISSION REGULATION (EU) No 1259/2011 in milk and dairy products.

A second analysis aimed to identify multidimensional biomarkers using a multiblock algorithm allowing the integration of multiple datasets in the growth model of preterm infants. The preliminary results did not suggest an impairment of preterm growth associated to the milk concentrations of POPs. The multiblock approach however revealed complex interrelated molecular networks of POPs, lipids, metabolites and amino acids in breastmilk associated to preterm infant growth, supporting the high potential of biomarkers exploration of this proposed workflow. Whereas the present study intended to identify simultaneously pollutant and nutrient exposure profiles associated to early preterm infant growth, this workflow may be easily adapted and applied to other matrices (e.g. serum) and research settings, favouring the functional exploration of environmental determinants of complex and multifactorial diseases.

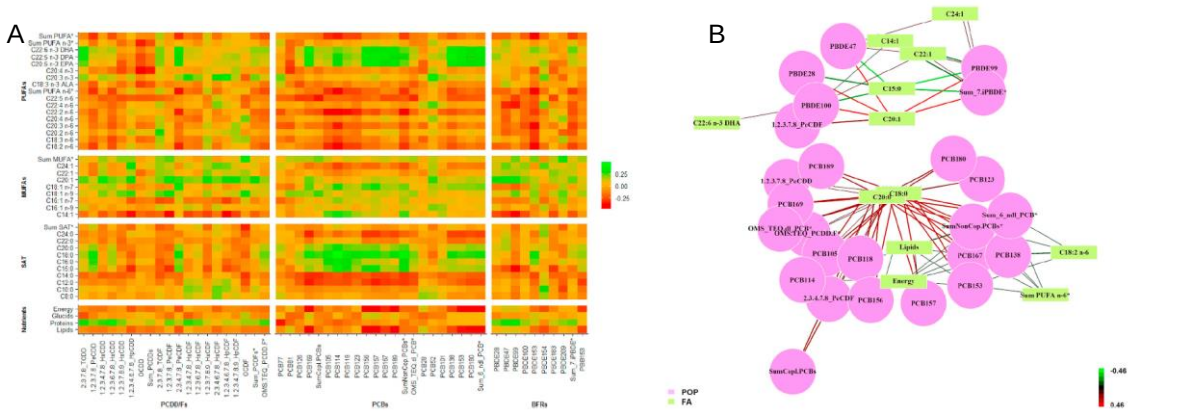


Figure 18. Heatmap representing the bivariate correlations between macronutrients, saturated (SAT), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids with persistent organic pollutants in breast-milk. (Panel A) The Spearman correlation coefficients are represented by the colour scale. The grouped variables as sum of individual concentrations are identified by an asterisk. Relevance network plots representing the strongest associations between fatty acids (green boxes) and pollutants (purple boxes) using sparse partial least squares. The strength of the correlations between pollutants and fatty acids is represented by colour key of edges (Panel B). The threshold of correlations was set up at −0.28 (negative associations, blackgreen) and 0.28 (positive associations, red) in order to highlight the most relevant clusters.

The results of this study stimulated the LactOMICS project 2021-2024 ("Breast Milk: An Environment-Sustainable Food System-Health Interface", funded by the SYALSA metaprogram from INRAE AlimH department grant call 2021). The LactOMICS project aims to implement an integrative approach based on food systems and their sustainability in the service of child health (Figure 19). This "fork-to-farm" approach has the sub-objectives of characterizing 1) the impact of the nutritional, microbiological and chemical composition of breast milk on the postnatal development of the child in order to identify biomarkers of benefits but also potential risks; 2) the nutrient-microbiota-contaminant interaction in breast milk; 3) the modulation of the nutritional, microbiological and chemical composition of breast milk by maternal dietary habits and their sustainability. Hence, the ambition of this project is to extend the preliminary analysis conducted in breastmilk, integrating novel targeted analysis of non-persistent pollutants, an exposomic non-targeted analysis, a characterization of milk microbiota and extending the nutrimetabolomic analysis.

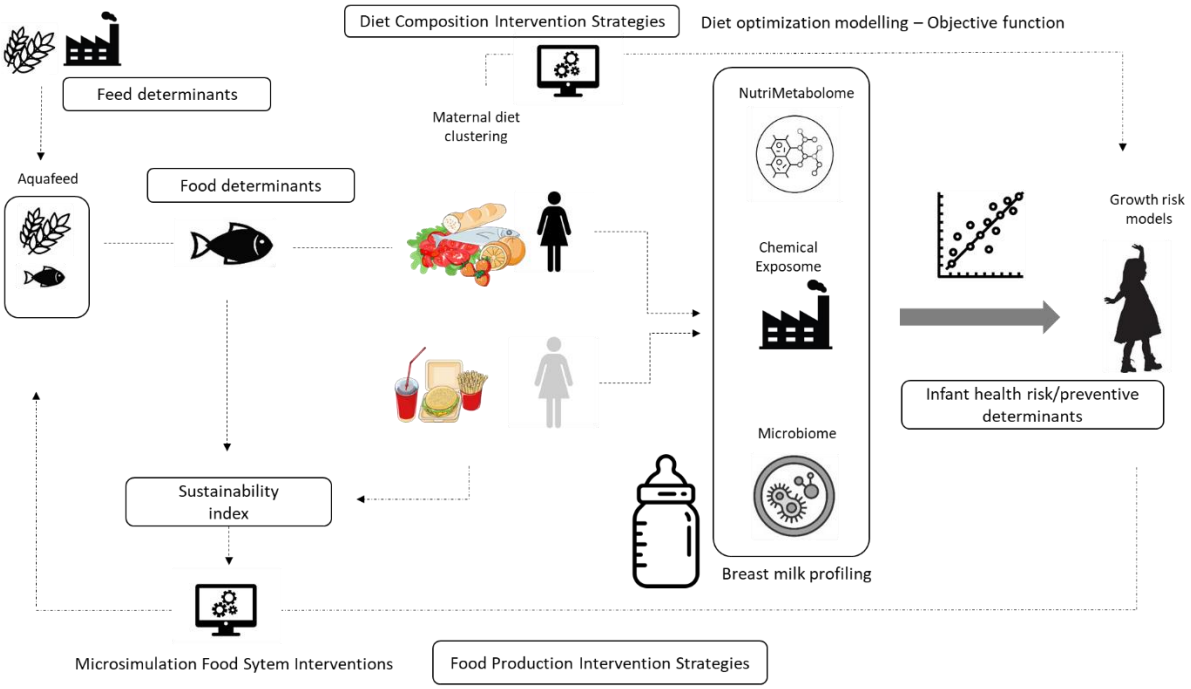


Figure 19. Schema of LactOMICS project concept

2.2 Internal exposure of women to POP mixtures during early/middle adulthood and reproductive health

The high hydrophobicity of POPs prevents their elimination from human bodies, accumulating during the entire life, with exception of women that exhibit major discharges during child deliveries and subsequent breastfeeding, as discussed above. Hence, the reproductive age of women become a critical window in terms of chemical exposure, due to their potentials impacts on female reproductive health, but also their offspring. National biomonitoring programs have shown the widespread exposure of women to POPs, yet few studies have targeted subpopulations with clinical vulnerabilities and disorders.

The EndoxOMIC-β project concept

In order to gain insight into the chemical exposure of young women and assess its impacts on reproductive health outcomes, we have conceived the EndoxOMIC-beta project built on a previous collaboration between the LABERCA Unit and the Nantes University Hospital (Endomet Project). The overall aim of this novel project was to assess the internal chemical exposure of women with different reproductive conditions, and pilot novel analytical (e.g. metabolomics) and statistical methods to gain insight on the underlying mechanisms linking the exposures and the reproductive phenotypes. The overall design of the study is depicted in Figure 20, including a clinical recruitment from two different sources (Gynecology-Obstetrics and Biology and Reproductive Medicine, Medically Assisted Reproduction Center), sharing the same clinical protocol. This recruitment design allowed to cover a wide range of reproductive disorders, including endometriosis (surgically diagnosed), polycystic ovaries, ovarian insufficiencies and other disorders causing infertility, in addition to fertile women. The overall project was selected for funding by the regional research grant call "Etoiles Montantes" in 2019 (Pays de la Loire Council), becoming my first research project as principal investigator. The project was executed with two doctoral students, Komodo Matta and Tiphaine Lefebvre, addressing the endometriosis and in vitro fertilization outcomes, respectively. The preliminary results of the EndoxOMICS-β project allowed to consolidate a partnership with the lead team on endometriosis epidemiology (Lead M. Kvaskoff, Exposome et Heredité, Centre de recherché en Epidémiologie et Santé des Populations, INSERM) and computational metabolomics (Lead F. Jourdan, Toxalim, INRAE) to scale-up the approach in a large population-based design (EndoxOMICS Project, grant call Fondation pour la Recherche Medicale 2022-2025).

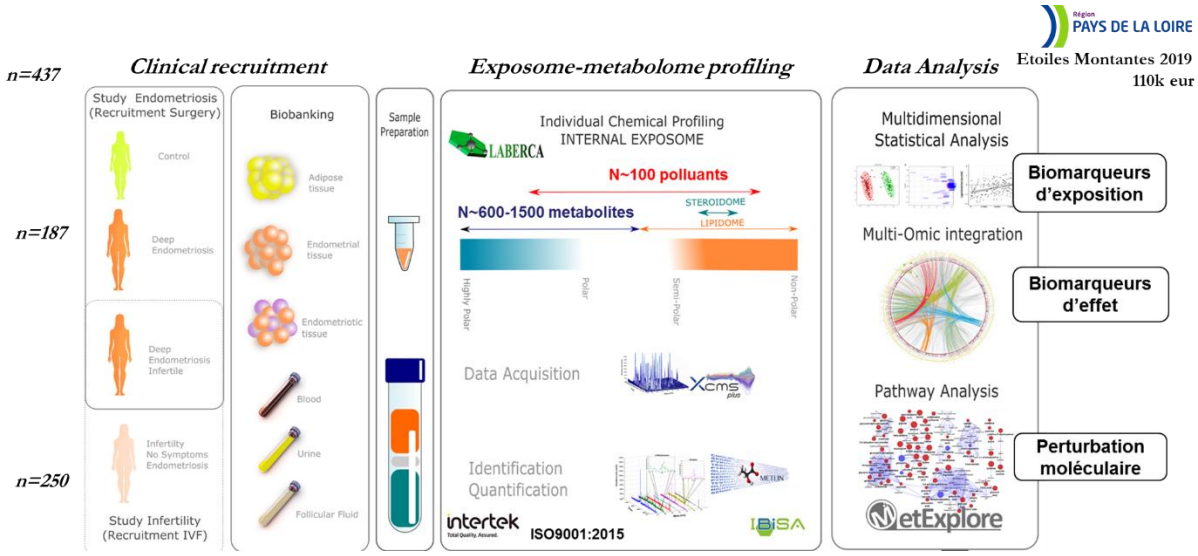


Figure 20. Schema of EndoxOMICS-β project

2.2.1 Associations between POPs and endometriosis: A multiblock approach integrating metabolic and cytokine profiling

Endometriosis is an estrogen dependent and multifactorial disease with an etiology still poorly understood and affecting around 10% of women worldwide. In addition, the disease is characterized with a long diagnostic delay of 7 years on average, for that reason, the identification of early diagnostic and prognostic biomarkers has become a priority in clinical research of endometriosis. Infertility and pain are common associated symptoms affecting women with endometriosis. The gold-standard experimental models of endometriosis are based on non-human primates, for instance Rhesus monkeys, that can menstruate and spontaneously present endometriosis. Alternative models include rodents with surgically induced endometriosis that may be limited to fully recapitulate the pathophysiology of endometriosis.

The application of 'exposome' concept appears as a good opportunity to overcome longstanding research gaps in etiological research or endometriosis. First, the holistic exposomic designs may better capture the multifactorial nature of the environmental of women with endometriosis, accounting for complex chemical mixtures and interactions with other external factors (e.g. diet). Second, applying Omic technologies (e.g. metabolomics) in observational research may allow gaining insight on the underlying mechanisms and supporting the biological plausibility of epidemiological associations overcoming the limits of experimental research. Finally, the use of Omic-based technologies to screen endogenous makers associated with endometriosis may also reveal potential biomarkers discriminating the diseases phenotypes with potential applications for early diagnostic biomarkers.

Through a comprehensive systematic review of in vivo and in vitro studies we found that inflammation and extracellular matrix remodeling pathways were major KE linking POPs and endometriosis, details in Figure 21 (Matta et al. 2021). The highly active 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was by far the most studied pollutant in association with endometriosis, with limited studies on the rest of POPs individually or as mixtures.

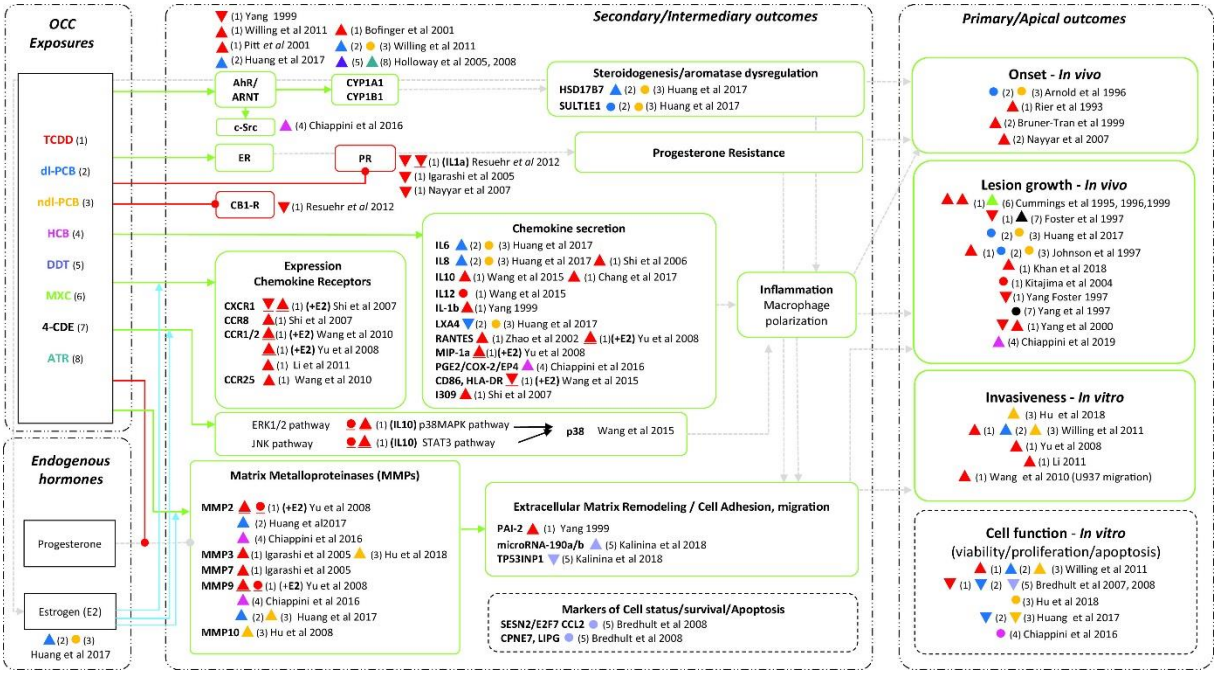


Figure 21. Network evidence plot summarizing the associations between exposure to organochlorine chemicals (OCCs) and the different end points related to endometriosis. Triangles and circles summarize the overall effect direction of the tested end point relative to the control. The triangle indicates significant increase, upside-down triangle

significant decrease and the circle no significant effect. The colored number key in parenthesis identifies the specific OCC indicated in the "Exposure box," also identified by the number in parenthesis [e.g., "red triangle (1)" indicates significant increase for TCDD]. In case of coexposure (i.e., with E2), the symbol appears underlined. Connecting lines ending with arrows indicate the overall evidence suggests up-regulation/increase, whereas connecting lines ending with circles indicate down-regulation/inhibition. Solid blue arrows indicate the presence of interactions, and dotted gray arrows stand for inferred associations between end points on the basis of current knowledge. The contour of end point boxes relates to the overall direction of effect toward positive associations (e.g., continuous line) or no conclusive effect (broken line). Note: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; ATR, atrazine; CB1-R, cannabinoid receptor type 1; CCR, chemokine receptor; COX, cyclooxygenase; CYP1A1, cytochrome P450; ER, estrogen receptor; HCB, hexachlorobenzene; IL, interleukin; LX, lipoxin; MXC, methoxychlor; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PCB, polychlorinated biphenyl; PGE, prostaglandin E; PR, progesterone receptor.

Hence, we conducted a study to proof the methodological applicability and discovery potential of integrating ultra-trace mass spectrometry profiling of POP biomarkers and endogenous biomarker profiling (MS metabolomics and cytokines) in a case-control study for the etiological research of endometriosis (Matta et al. 2022). The approach was applied in a pilot clinical-based study conducted in France where women with and without surgically confirmed endometriosis were recruited. Serum samples were analysed with high-resolution MS for about 30 PCBs, OCPs and PFAS. About 600 serum metabolites and lipids were identified with targeted metabolomics using tandem MS with the Biocrates MxP® Quant 500 Kit. A panel of 4 pro-inflammatory cytokines were analysed using ELISA-based 4-PLEX analyser. Statistical analysis included a battery of variable selection approaches, multivariate logistic regression for single-chemical associations, BKMR to identify mixture effects of POPs and a multiblock approach to identify shared biomarker signatures among high-risk clusters (details in Figure 22).

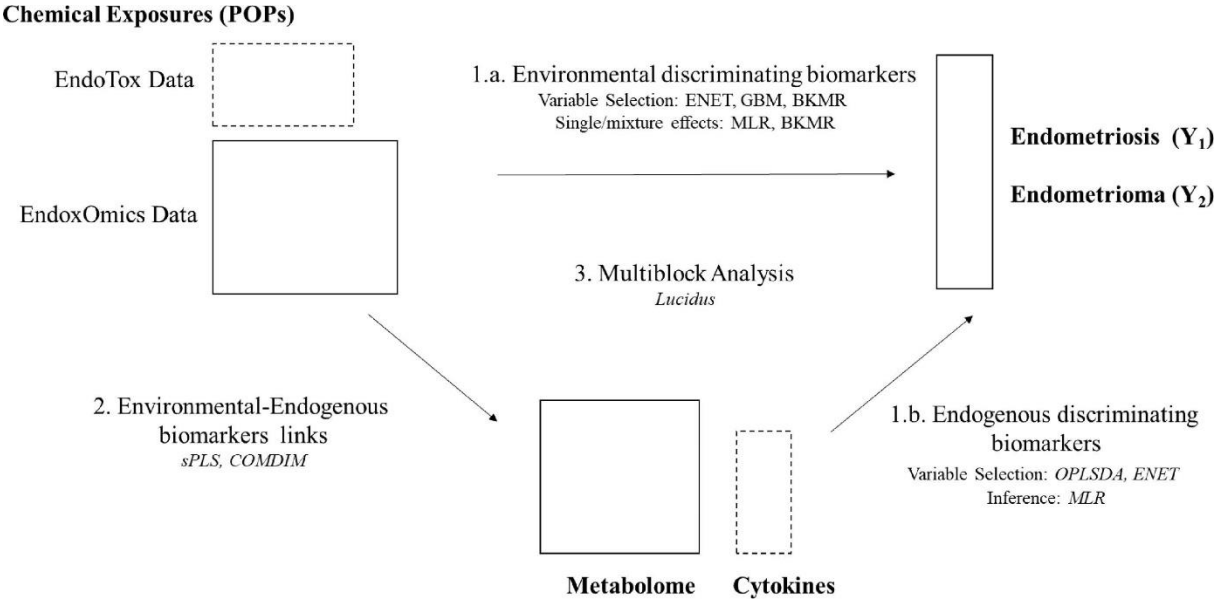


Figure 22. Statistical workflow diagram. Path 1a and 1b (Single Block) focuses on the associations between each individual block and the outcome (Y). Path 2 (Interblock) focuses on the correlation between the exposome (E) and the metabolome (M). Lastly, Path 3 (Multiblock) integrates both E and M blocks in relation to Y. Boxes with doted borders depict datasets with incomplete observations (e.g. Cytokines) or biomarkers (e.g. EndoTox Data) for the pooled analysis. Methods used in each path are identified with abbreviations in Italics: BKMR, Bayesian Kernel Machine Regression; ENET, elastic-net regression; COMDIM, common dimension analysis; GBM, gradient boosting-machine; LUCIDus, latent unknown clustering with integrated data; MLR, multivariate logistic regression; OPLS, orthogonal partial least squares; sPLS, sparse partial least squares.

The results showed positive associations between some POPs and endometriosis risk, including the pesticide trans-nonachlor Odds Ratio (95% Confidence Interval) 3.38 (2.06–5.98), $p < 0.0001$ and PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$. The BKMR approach showed a tendency

of a positive cumulative effect of the mixture, however trans-nonachlor exhibited significant associations within the mixture and interacted with other PCBs, strengthening the effects at highest concentrations. Finally, the multiblock analysis, relating the various blocks of data, revealed a latent cluster of women with higher risk of endometrioma presenting higher concentrations of trans-nonachlor, PCB 114 and dioxin-like toxic equivalents from PCBs, together with an increased inflammatory profile (i.e. elevated interleukin-8 and monocyte chemoattractant protein-1), Figure 23. It was also highlighted a specific metabolic pattern characterized by dysregulation of bile acid homeostasis and lipase activity. Further research will be required with larger sample size to confirm these findings and gain insight on the underlying mechanisms between POPs and endometriosis.

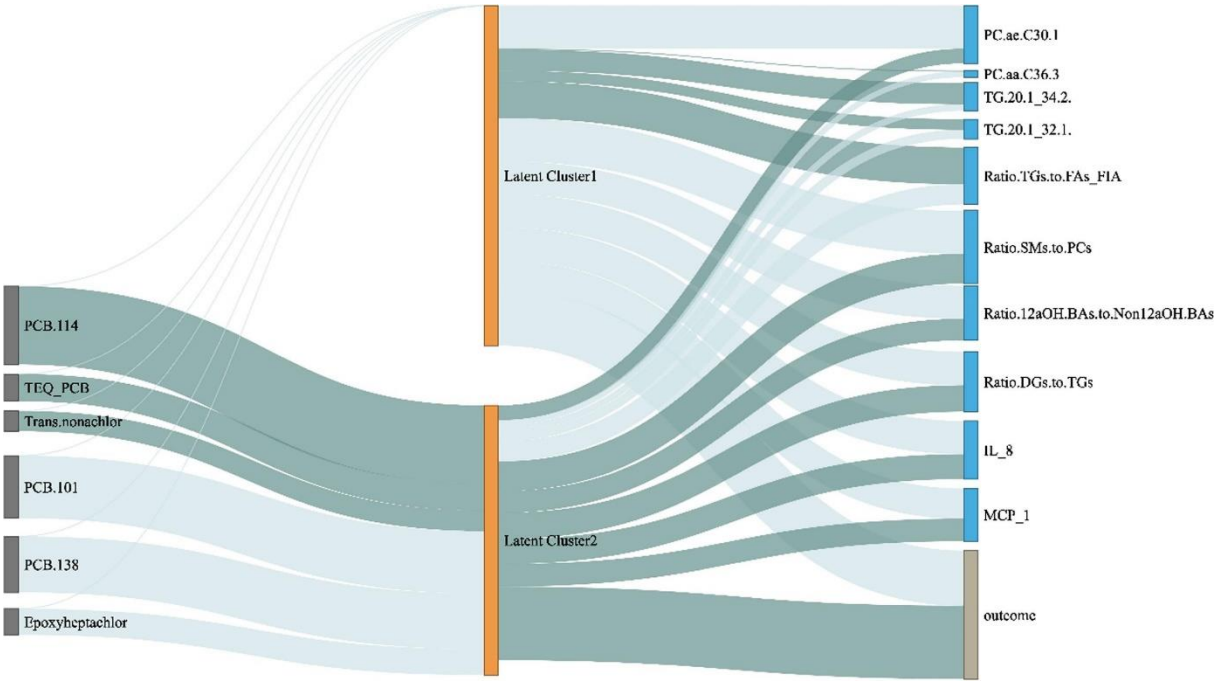


Figure 23. Sankey diagram of the secondary structural integrated analysis of POPs exposure and individual lipid metabolites and cytokines in serum for the identification of latent variable clusters associated with severe cases of endometriosis (OMA). Dark grey bands represent positive associations and light grey bands indicate negative associations, with the width proportional to the size of the association. POPs exposures and cytokines are positively associated to Latent Cluster 2, which contributes positively towards outcome (OMA).

2.2.2 Associations between POPs, ovarian function and in vitro fertilization outcomes

Despite the widespread presence of POPs among general population, including women of childbearing age, little is known about the impacts of individual POPs and their mixtures on female infertility and IVF outcomes. In a systematic review and evidence map of epidemiological studies we showed that main families of POPs are still pervasive in follicular fluid and serum of women undergoing IVF treatments. Globally, we found inconsistent findings across studies for specific exposure-outcome dyads, suggesting that adverse effects of POPs on IVF outcomes cannot be ruled out. Specifically, there is evidence that POPs, notably some polychlorinated biphenyls and organochlorine pesticides, may impair embryo quality and pregnancy rates. Most studies have been performed in small cohorts (n<50) and focused on PCBs and OCPs, whereas major research gaps remain for emerging compounds (e.g. PFAS) and the most clinically relevant outcome, live birth rate (Figure 24). The overall evidence presented 'serious' or 'very serious' risk of bias, mainly due to the lack of consideration of relevant confounding variables, low sample size or underreporting of methods. Globally, we judged the level of evidence being "low". Given the high economical and societal costs associated to infertility and IVF, further well-designed research is urged to fill the highlighted gaps (Lefebvre et al. 2021).

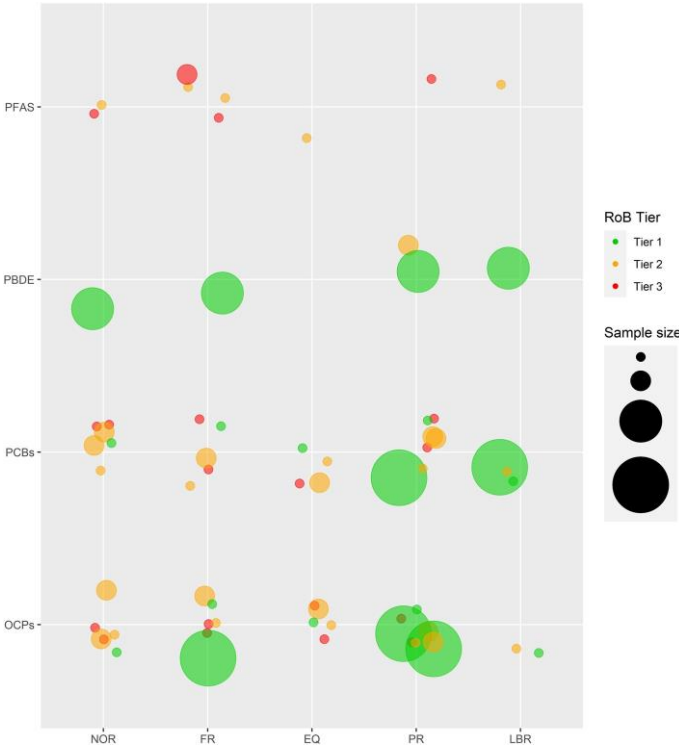


Figure 24. Evidence gap map summarizing the size and risk bias tier classification for included studies assessing the relationships between the main POP families and in vitro fertility outcomes. Each bubble represents a single study with size proportional to the population included in the analysis and colour related to the respective risk of bias Tier classification according the NTP/OHAT approach. Tier 1 refers to those studies classified as "unlikely", Tier 2 as "serious" and Tier 3 "very serious" risk of bias.

On that basis we have conceived an observational and hospital-based study aiming 1) to characterize the internal exposure levels of PCBs, OCPs and PFAS in women undergoing IVF; and 2) evaluate their association with markers of ovarian function and IVF outcomes. Hence, women (n=136) attending the Assisted Reproductive Technology service from Nantes University Hospital (France) were prospectively recruited. Serum samples were analysed using liquid chromatography with tandem-mass spectrometry for 14 PFAS. Follicular fluid was analysed with gas chromatography coupled to high resolution mass spectrometry for 30 PCBs and 14 OCPs. Ovarian function markers, biological and clinical IVF outcomes were ascertained by embryologists or clinicians using standardized protocols. The associations were assessed with multivariate regression models and BKMR. Twenty-seven POPs were frequently quantified in almost all women attending IVF. Positive associations were found between the pesticides hexachlorobenzene, β-hexachlorocyclohexane and epoxy-heptachlor with Follicular Output RaTe (FORT) (Lefebvre et al. 2022b). Our results globally showed that most POPs were not associated with clinically relevant

IVF outcomes, like live birth rates (Figure 25). Nonetheless, negative associations between PCB138 and trans-nonachlor with the number of usable blastocysts were observed, β -0.28 (95%CI [-0.52; -0.04] p=0.02) and β -0.22 (95%CI [-0.40; -0.03] p=0.02 (Lefebvre et al. 2022a). Altogether, our results indicate a lack of associations between most individual chemicals or their mixtures with markers of ovarian function and IVF outcomes. Nonetheless, some highly prevalent OCPs were linked with markers of ovarian responsiveness like FORT and the number of usable blastocysts, supporting the need of conducting further studies in a larger populations sample in order to ensure sufficient statistical power to identify mild effects and a robust stratification analysis to account for the large underlying disease heterogeneity.

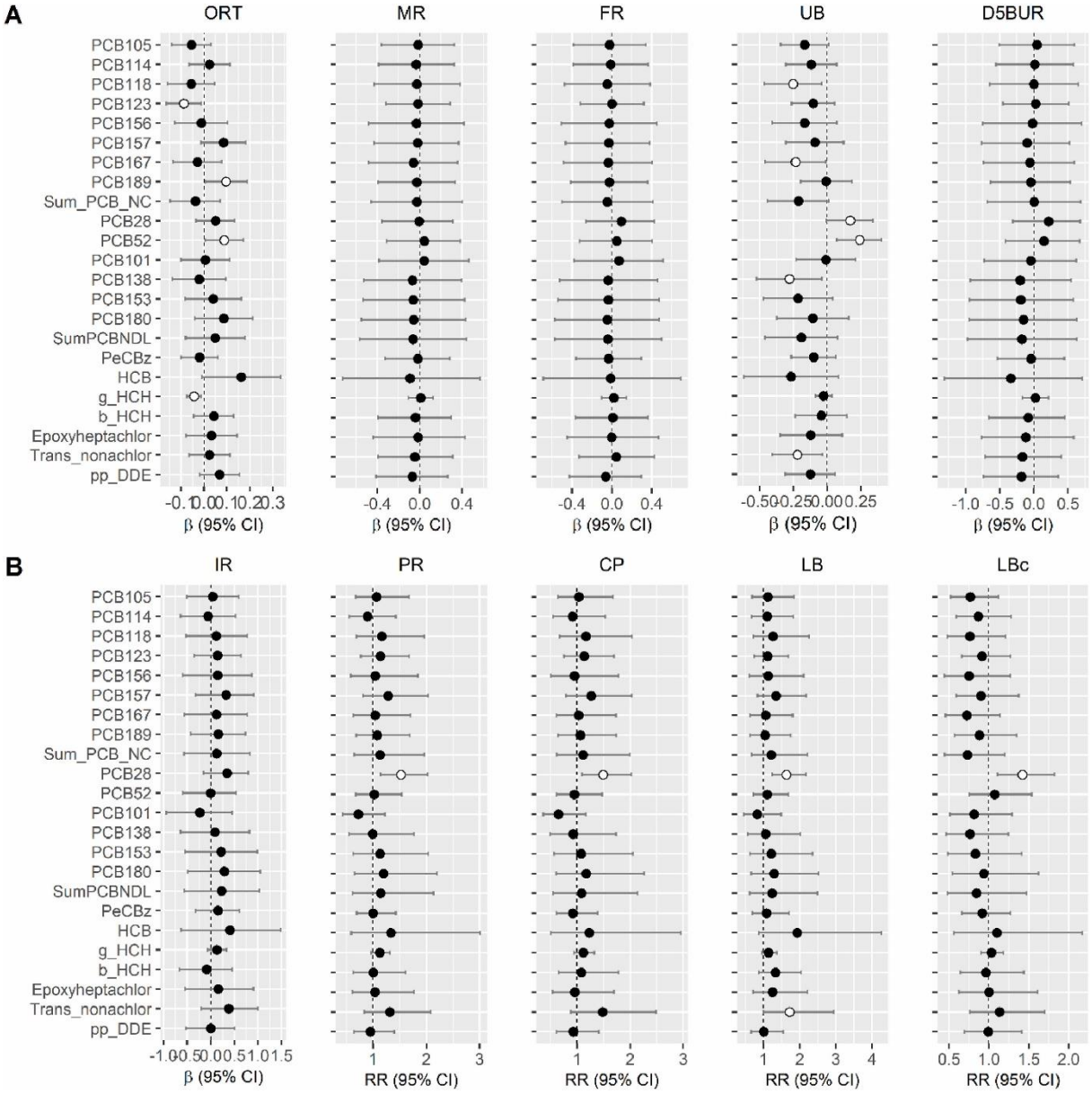


Figure 25. Forest plots depicting the associations between organochlorinated chemicals in follicular fluid with in vitro fertilization (IVF) outcomes. Associations are represented by their beta coefficients (β) and relative risks (RR) with 95% confidence intervals (CI) from models adjusted for age, body mass index and the levels of anti-Müllerian hormone. Intermediary outcomes (Panel A) included number of oocyte retrieved (ORT), oocyte maturity rate (MR), fertilization rate (FR), number of useable blastocyst (UB) and day 5 useable blastocyst rate (D5BUR) and clinical outcomes (Panel B), implantation rate (IR), pregnancy rate (PR), clinical pregnancy rate (CP), live birth rate after 1st blastocyst transfer (LB), cumulative live births (LBc). Statistically significant ($p < 0.05$) associations are depicted with white circles.

2.3 Exposure of women to POP mixtures during late adulthood and breast cancer

Internal exposures assessment of women at late adulthood and elderly, reflects the cumulative exposure fingerprint resulting from multiple decades, spanning towards critical exposure periods when legacy chemicals were produced at high volumes. For instance, women at the 60s (born at the 1960s) were heavily exposed to HCB during over three decades until its restriction in France in 1988. I have participated in two collaborative projects where the exposure of elder women to POPs were the focus of research, especially in relationship with breast cancer (BC) risk (ED-Cancer project, Lead F. Mancini, INSERM, Centre de recherche en Epidémiologie et Santé des Populations) and breast cancer metastasis (METAPOP project, Lead X. Coumoul, INSERM UMR-S 1124).

POPs and breast cancer risk

The main objective of the **ED-Cancer project** was to estimate the long-term risk of HRC associated with chronic exposure to PFASs and BFRs as selected targeted EDCs, in the E3N cohort. The E3N (Etude Epidémiologique auprès de femmes de l'Education Nationale) is a prospective cohort study involving 98,995 French women, aged 40-65 years at inclusion in 1990 and insured by a national health insurance plan mostly covering teachers (Mutuelle Générale de l'Education Nationale, MGEN). Ultra-trace analysis of PFAS and BFRs was conducted in blood from 194 BC cases and 194 control women, identified using a nested-case control design (Mancini et al. 2020a; Mancini et al. 2020b). The results showed positively linear associations between PFOS concentrations and the risk of ER+ (3rd quartile: OR = 2.22 [CI = 1.05-4.69]; 4th quartile: OR = 2.33 [CI = 1.11-4.90]); p-trend = 0.04) and PR+ tumors (3rd quartile: OR = 2.47 [CI = 1.07-5.65]; 4th quartile: OR = 2.76 [CI = 1.21-6.30]; Ptrend = 0.02) (Mancini et al. 2020a). When considering receptor-negative tumors, only the 2nd quartile of PFOS was associated with risk (ER-: OR = 15.40 [CI = 1.84-129.19]; PR-: OR = 3.47 [CI = 1.29-9.15]). In turn, the analysis of ¨PBDES suggested no clear association between internal levels of PBDEs and PBB-153 and the risk of breast cancer in postmenopausal women (Mancini et al. 2020b). The BKMR multipollutant models suggested a negative cumulative effect of BFR and PFAS on ER- breast cancer risk, and a positive cumulative effect on ER + breast cancer risk (Frenoy et al. 2022b). The analysis of dietary exposure to PBDEs of the entire E3N cohort (5 686 incident cancers) showed a mild positive association with BC risk -trend = 0.06, HR_{Q5vsQ1} and 95% CI: 1.09 [0.99;1.20]). Interestingly, a dietary effect modification was identified with vegetable oil consumption, being the high consumers at highest risk (e.g. ≥4.6 g/day (HR_{Q5vsQ1} and 95% CI: 1.23 [1.08; 1.40]) whereas no effect was observed among low consumers (HR_{Q5vsQ1} and 95% CI: 0.97 [0.86; 1.10]) (Frenoy et al. 2022a). These results further support the role of diet and nutrients on the characterization of chemical exposure-health associations (Cano-Sancho and Casas 2021; Cano-Sancho et al. 2023)

POPs and breast cancer aggressiveness

The impact of POPs on breast cancer aggressiveness was addressed within the METAPOP project, using a hospital-based case-control study including 91 French women aged 62 years old in average (range 37–93) with breast cancer, 38 of them presenting a metastasis. Women were recruited the Department of gynecological-oncological surgery of the Georges-Pompidou European Hospital (Paris, France) from 12/2013 to 11/2017. A comprehensive characterization of over 60 POPs in adipose tissue biopsies and paired serum samples was conducted, including 20 PCDD/Fs, 20 PCBs, 7 BFRs and 30 OCPs. We found that most POPs (59 congeners) were present in women fat and serum (Figure 26), at concentrations around 2 fold higher than in French women aged 30 (Ploteau et al. 2016). Strong positive correlations (rho > 0.6) were observed between most PCDD/Fs and PCBs with some minor exceptions showing mild or no correlations, such as OCDF or 1.2.3.4.7.8.9 HpCDF (Benoit et al. 2022; Koual et al. 2019). Interestingly, the congeners from

groups of PBDEs were not correlated with the rest of POPs, except for PBB153 that correlated with PCBs (180 and 189), (Figure 13B). A similar correlation pattern of POPs in adipose tissue was observed among the group of patients with metastatic and non-metastatic breast cancer as previously published (Koual et al., 2019). Concentrations of dioxin-like toxic equivalents (TEQ) were slightly higher among women with non-metastatic cancer, who in turn, presented lower concentrations of non-dioxin-like PCBs and brominated flame retardants (BFRs) (Fig. 3A-C). The relative abundance of congeners was similar among cancer groups for most congeners except for PBDE209 and PBDE47, slightly higher and lower in the metastatic group, respectively (Fig. 3C).

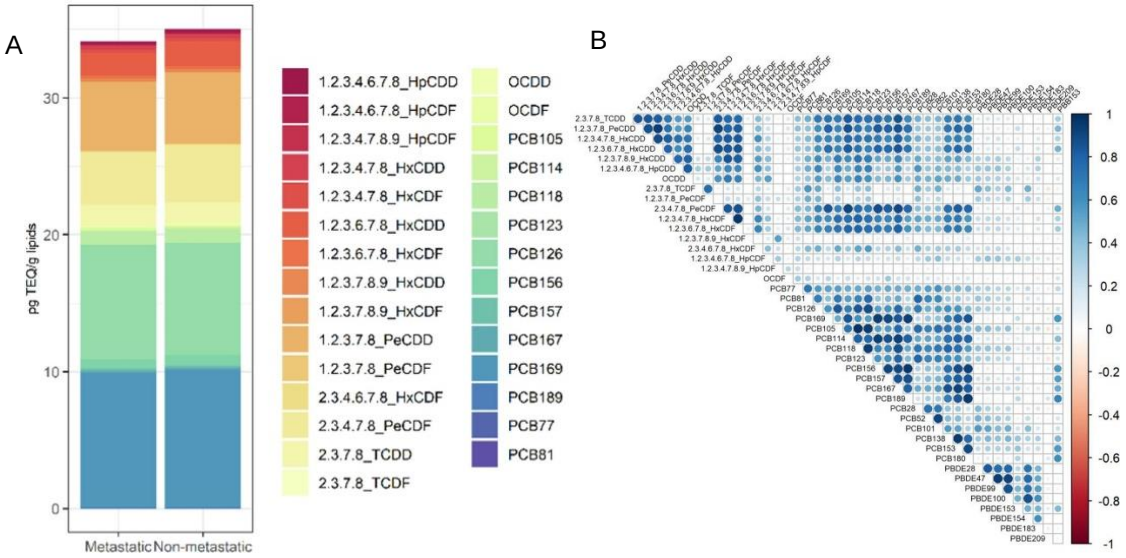


Figure 26. Concentrations of chemicals in adipose tissue from patients with metastatic and non-metastatic breast cancer for toxic equivalents (TEQ) in panel A. Heatmap displaying the Spearman coefficients for the bivariate correlations between chemicals (B).

The results from single pollutant models showed that 2.3.7.8-TCDD concentrations in AT were positively associated with the risk of metastasis in 43 patients who have BMIs equal or higher than 25 kg/m2 (odds ratio: 4.48 (1.32-20.71)). Furthermore, the concentrations of 2.3.7.8-TCDD and two coplanar PCBs (77&169) in AT also were positively associated with the risk of lymph node metastasis and the tumor size (Koual et al. 2019). The multipollutant analysis using a BKMR and weighted quantile sum regression (WQS) models did not showed statistically significant results when all women were considered (Benoit et al. 2022). However, among patients with a BMI > 22 kg/ m2, our mixture was positively associated with tumor size (OR: 9.73 95 %CI: 1.30-18.15) and metastasis (OR = 3.98 95 %CI = 1.09-17.53) using the WQS model. Moreover, using the BKMR model on chemical families, dioxin like chemicals and PCDD were associated with a higher risk of metastasis.

We are currently conducting further analysis with the full transcriptomic profiles in order to gain insight on the potential mechanism underlying those associations

2.4 Partitioning of POPs between tissues and blood: implications in exposome-health studies

Blood is the most widely used matrix for biomonitoring of persistent organic pollutants (POPs), because under the assumption that POPs are homogenously distributed within body lipids at steady state, blood levels may reflect the concentrations of more invasive tissues such as adipose tissue. However, the equilibrium among compartments rarely occurs in real-life. In a comparative study of biomarker-based models using different ways of consider blood biomarkers and compared to the referent adipose tissue based models we showed that despite the high correlations, different conclusions could be reached in the case study of endometriosis due to the low concordance or risk estimates (Cano-Sancho et al. 2018).

Actually, a list of list of analytical, physicochemical and biological factors may contribute to the variability of partitioning of POPs between lipid compartments with potential impacts on the use and interpretations of circulating biomarkers, as we discussed elsewhere (Cano-Sancho et al. 2020b). Among those, the metabolic disrupting effect of many POPs were highlighted as a potential source of variability due to their capacity to target and interact with major processes and organs involved in their pharmacokinetics, summarized in Figure 27.

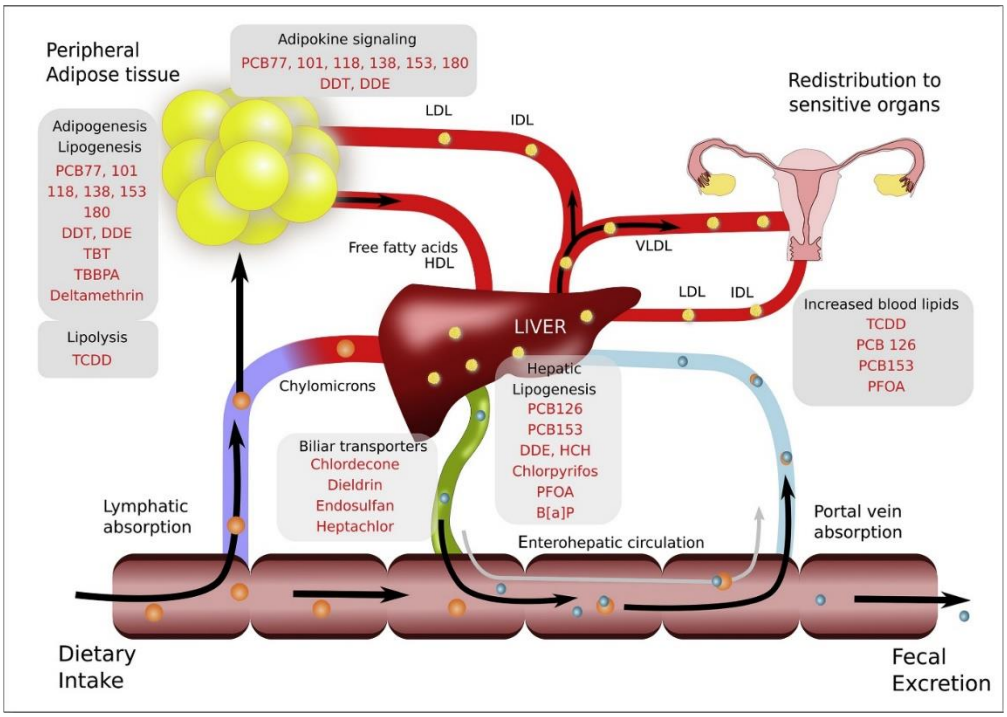


Figure 27. Schematic representation of LEDCs distribution system, with adipose tissues as main storage depot and blood as main vehicle, all orchestrated by liver to satisfy the energy demands. In gray boxes appears the main metabolic endpoints directly linked to LEDC distribution and that has been reported to be impaired by LEDCs (examples of obesogenic LEDCs in red) (Cano-Sancho et al. 2020b).

We also conducted a narrative literature review of human observational studies reporting concentrations of POPs in paired samples of adipose tissue with other lipid-based compartments (e.g., serum lipids). A meta-regression was performed to identify factors responsible for variability. All included studies reported high variability in the partition coefficients of POPs, mainly between adipose tissue and serum. The number of halogen atoms was the physicochemical variable most strongly and positively associated with the partition ratios (Figure 28A), whereas body mass index was the main biological factor (Figure 28B), positively and significantly associated (Moriceau et al. 2022).

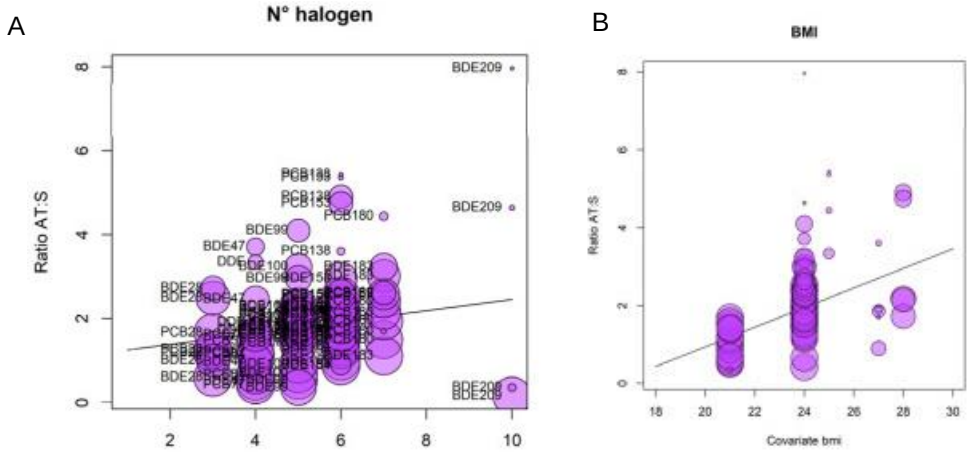


Figure 28. Bubble plots representing the associations between the mean partitioning ratios of persistent organic pollutants between adipose tissue and serum reported in the literature and number of halogen atoms (A) and body mass index, BMI (B)

Partition of POPs between serum and follicular fluid

Follicular fluid is contained in the follicles during their final growth to support the maturation of oocytes. The chemical composition is the result of transfer of blood constituents through the follicular barrier and the secretory activity of the granulosa and theca cells, thus susceptible of being contaminated with exogenous compounds. Up to date, few studies have explored the partitioning of POPs between blood and follicular fluid, focusing of few chemicals. Hence, during the framework of the EndoxOMIC-β project we conducted an exploratory study to gain insight on the chemical composition of that poorly investigated biological matrix. We conducted a comprehensive analytical study involving women aged 18 to 43 years, referred for IVF to the ART center of the University Hospital of Nantes, France. Finally, 19 women were considered for the comparison of distributions of POPs, but additionally 10 of them were also considered for the paired comparison of metabolic profiles. The content of 14 PCBs and 25 OCPs was measured in paired samples of serum and follicular fluid using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS). Overall, PCBs were detected in all serum and follicular fluid samples with significantly higher detection limits than in serum. Concerning pesticides, 4 compounds (HCB, β-HCH, epoxyheptachlor, p,p'-DDE) were detected in all samples and both matrices. For all compounds with a percentage of detection higher than 50% in both matrices, the concentrations were 2-3 fold higher levels in serum than in follicular fluid. For several pesticides like PeCBz and oxychlordane the detection rates were higher in follicular fluid, being the median concentrations of PeCBz was highest in serum. The partition coefficients of POPs between the two matrices were positive for all compounds, with a median rank between 1.6 (PeCBz) and 4.4 (PCB114). PCBs (Figure 29A) generally showed higher coefficients than pesticides (Figure 29B). The highest coefficient was noted for γ-HCH among the pesticides, and PCB189 among the PCBs. The correlation of POPs between the two matrices (i.e. serum and follicular fluid) is presented in Figure 29. Most of molecules were strongly and positively correlated between the two matrices with Spearman coefficients (ρ) between 0.36 (i.e. PCB123) and 0.90 (PCB180 and β-HCH), except for g-HCH, with a weak correlation (ρ=0.14) and α-HCH, negatively correlated (ρ=0.49) (Lefebvre et al 2023, Article under preparation).

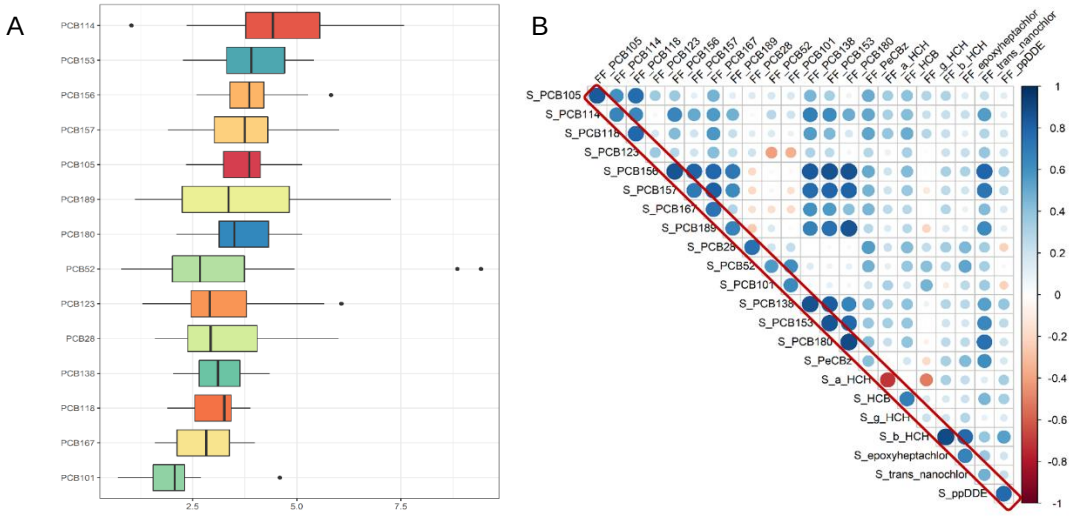


Figure 29. Boxplot of ratios (A) and correlation (B) of POPs between serum and follicular fluid (FF)

3. SCIENTIFIC RESEARCH PERSPECTIVES

3.1 Exposome research to decipher the role of environmental chemical insults on female reproductive health

The exposome concept was first proposed by time by Wild in 2005 (Wild 2005), referring to all the influences that an individual is subjected to during its lifespan, including chemical, biological and psycho-social agents, and depend on several factors as diet, personal habits, habitats, etc. The term has been redefined over the years, also including in its definition the biological response induced by these environmental exposures (Vermeulen et al. 2020). The exposome encompasses a wide range of exposures, among which the study of the chemical exposome addresses the influence on health of exposure to chemical mixtures through human lifespan. This framework has large potential to support the identification of complex exposure-disease relationships involving mixtures of environmental chemicals. In addition, among the chemical chemistry community "exposomics" has been growing even as a novel discipline, with the ambition of holistically providing comprehensive information on both, biomarkers of exogenous chemical exposures and their internal biological response, rather than just a list of targeted compounds traditionally done under hypothesis-driven studies (Sillé et al. 2020). The exposome framework and the novel exposomic (and other omic) analytical advances provide a ground-breaking landscape to better understand the interaction between chemical environment and human health. For that reason, I have the ambition of contributing to gain insight on the research gaps arising the potential links between environmental chemical mixtures and female reproductive disorders, implementing the exposome principles. To do that, I will focus the next few years on implementing existing tools in applied studies and contributing to develop new strategies for data and chemical analysis (Summary of ongoing and submitted project in Table 2):

Applied studies

During the last years, I have identified that the impact of environmental chemicals on female reproductive disorders remains a major gap and up to date scarcely addressed in environmental epidemiology. Hence, I have the ambition of contributing to the knowledge on female hormone-dependent disorders such as endometriosis, infertility or female hyperandrogenic spectrum disorders such as early puberty and polycystic ovaries.

The proof-of-concept study on the integration of metabolomics to support the research between POPs exposure and endometriosis, provided the rationale to scale-up the methods in a population-base nested case-control study within the E3N French cohort funded by the Medical French Foundation (FRM). This collaborative project will be conducted between 2022 and 2024 with leading partners on the domain of epidemiology of endometriosis (INSERM, M. Kvaskoff) or systems metabolomics (ToxAlim, INRAE, F. Jourdan). This study will represent the largest population-based and first prospective study ever conducted to date on endometriosis and POPs, allowing the accurate and robust identification of complex mixtures of POPs as risk factors for endometriosis. The design is innovative and very robust, being the first prospective study in the field preventing reverse causation.

The differential exposure profiles of POPs observed among women with different manifestations of endometriosis will be further explored in the collaborative POPENDO project lead by the Exposome and Heredity team (Lead M. Kvaskoff, CESP, INSERM). The main aim of the project is to explore patient characteristics modulating the links between POPs and with endometriosis severity in the ComPaRe-Endometriosis cohort (n>12,000). The project has been pre-selected in the ANR2022 call, waiting list, pending of final results.

During the EndoxOMICS-β project we have consolidated a rich clinical hospital-based cohort and biocollection, including around 200 women with deep endometriosis and endometrioma with adipose tissue biopsies, but also around 350 women attending IVF that provided follicular fluid and serum. I would like to find the resources to analyze the adipose tissue from women with

endometriosis to characterize the content of POPs and the metabolomics profiles in order to gain insight on the cross-talk between adipose tissue dysfunction and endometriosis location and severity. This subject has been integrated as a work-package within the ENDOFAT project (Lead M. Koual, INSERM) currently under evaluation in the grant call ANR2023. Considering that only 136 women out of the 350 recruited in the IVF arm, I will develop a research grant proposal to complete the analysis of POPs, non-POPs and effect biomarkers in the entire cohort. Future perspectives concerning endometriosis research will involve the analysis of more emerging and non-persistent environmental pollutants such as bisphenols, phthalates or organophosphorus compounds.

I have the ambition of extending the application of developed exposomic-metabolomic framework to other female reproductive disorders scarcely explored such as female hyperadrogenism, manifested with metabolic disorders, dysregulation of hormone signaling, precocious puberty or polycystic ovaries. A novel methodological approach applied to hyperandrogenic disorder is presented in detail in the next section.

A major limiting factor to conduct applied epidemiological studies is the access of cohort data or biological resources. Up to date, I have developed a network of collaborations to facilitate the access to data of cohorts such as INMA (Spain) or HELIX (Europe) to ensure projects such as NutPOP and the perspective of PUBERPOP to gain insight on the links between POPs during the pre-natal period and early puberty (Data access accepted in 03/2023). Within the framework of PARC project I am also leading 3 projects within the Task 4.1.4.2 that will facilitate the data access to the unique European HBM4EU adult cohort to analyze the link between pesticides, UV-filters or acrylamide with overweight/obesity. As proposed in the next section, the data-access demand to Nutrinet-Santé cohort has been planned in a near future upon the advancements of funding requests. Likewise, the access to biological resources and robust epidemiological settings will be granted in the future with ongoing collaborations with epidemiological teams, hence accessing to established cohorts and biocollections such as E3N-E4N, ComPaRe or ELFE.

Methodological research

Different methodological gaps remain to be addressed during the next years in order to support the implementation of more efficient exposome-scale strategies in observational settings. Hence, I will contribute to identify those gaps and stimulate the research innovations, mainly to overcome the ongoing challenges on data analysis, exposure assessment and integration of biological/toxicological information. Concerning data analysis, the development and application of statistical methods for multipollutant analysis will still be of interest during the next years within the framework of ongoing European projects such as PARC (task 4.1.4.2), but also multiblock data integration (Doctoral thesis of E. BABIN and Collaborative Project MIMS granted by INRAE-DIGITBIO). I will contribute to the process of designing more efficient analytical methods to accommodate traditional strategies for biomonitoring and the needs of epidemiological research, including the miniaturization of analytical targeted methods, identification of novel matrices, integration of ad-hoc modelling strategies (PBPK or predictive models) or refining the pipelines to integrate non-targeted methods in epidemiological settings. Exposome research on environmental chemicals will also benefit of further integrating toxicological knowledge in epidemiological approaches. As further developed in the next section, the adverse outcome pathway framework and functional exposomics can contribute to develop such bridges and shape novel multidisciplinary approaches.

Table 2. Gantt chart depicting the on-going (green) and submitted (gray) projects

	2023	2024	2025	2026	2027
Applied projects					
Endometriosis					
EndoxOMICS project (PI, funded FRM2021)					
POPENDO project (WP leader, submitted to ANR2022)					
MACHENRO project (Co-lead/WP leader, submitted to HORIZON2024)					
Infertility /IVF outcomes					
EndoxOMICS-IVF-2 (Complete analysis IVF: ANR, ANSES 2024)					
Statistical analysis of EndoxOMICS-β					
Hyperandrogenism and PCOS					
EggXposome project (PI, submitted to ERC-CoG) (3 postdoc + 2 PhD students) to be submitted next ERC call					
PuberPOP project (Data requested INMA/HELIX approved) (Intend to 1 PhD student)					
Breast Cancer					
BCEXPOS Project (Partner, funded CGO Region 2021)					
Covid-2019					
CHEMVAX Project (WP Leader, ANSES 2023)					
Methodological projects					
Data analysis					
MULTIBLOC Project (Thesis supervisor E. BABIN)					
PARC Task 4.1.4.2 (Leader 3 projects: UV filters, pesticides)					
Multibloc Method Benchmarking MIMS Project (DIGITBIO)					
Exposure assessment					
LACTOMICS (co-PI, funded SYALSA INRAE2021)					
MycoEXPO Project (Thesis supervisor JA. GALLARDO)					
PARC project Task 4.3.4-8.2.1 (Partner)					

3.2 Deciphering the Interactions Between Environmental Dysmetabolism and Female Hyperandrogenic Disorders: A Novel Approach Based on Systems Exposomics

[This research proposal has been submitted to European Research Council – Consolidator Grant call - February 2023]

Declining fertility and female reproductive health/Life-course associated pathologies

The global trends of human population growth has experienced an alarming switch during the last 5 decades, paralleled by a persistent decline of fertility rates all across the globe, but especially among the most developed and industrialized countries (Aitken 2022). This declining of fertility rates has been paralleled by an increase of prevalence of female reproductive disorders such as PCOS or endometriosis, directly related with female subfertility and infertility. For instance, PCOS affecting 5-15% women and characterised by hyperandrogenism, ovulatory dysfunction and polycystic ovaries, has been recognized as the first cause of anovulatory infertility (Teede et al. 2010). The physiopathology of PCOS remains still largely unknown, yet it is well acknowledged that besides an endocrinopathy, it has also an underlying dysmetabolic background arising the disorder. Women with PCOS have high prevalence of for the high prevalence of overweight/abdominal obesity, dyslipidemia, non-alcoholic fatty liver disease, type 2 diabetes mellitus, and metabolic syndrome (de Medeiros et al. 2021). The functional ovarian hyperandrogenism is the fundamental defect in most PCOS due to an unexplained type of steroidogenic hyperactivity that seems to disturb the intraovarian processes that normally coordinate ovarian androgen and estrogen secretion (Rosenfield and Ehrmann 2016). Despite the close cross-talk between dysfunctional adipocytes, adrenocortical and ovarian steroidogenic cells has been acknowledged, their precise role on the causal chain of events leading to dysmetabolic infertility is poorly understood (Medenica et al. 2022). Noteworthy, half of women with PCOS have also associated irregular menstrual cycles, whereas some studies has also associated the early onset of puberty and low birth weight with later PCOS risk (Rosenfield and Ehrmann 2016). These interconnected pieces of evidence has stimulated to structure a common physiopathological origin of female reproductive disorders, coining the ovarian dysgenesis syndrome (ODS), as in utero programing of ovarian structure or function that may cause an impairment of reproductive parameters in adulthood. Hence, the ODS hypothesis proposes a common origin for a group of related female reproductive diseases and disorders, such as reduced fertility, polycystic ovary syndrome, premature ovarian insufficiency and reproductive-site cancers (Johansson et al. 2017). The concept of disease continuum from fetal growth to adulthood has been well conceptualized under the Developmental Origins of Health and Disease (DOHAD) hypothesis, yet the mechanisms remain poorly understood (Barker and Osmond 1986).

Emerging role of environmental obesogens on reproductive function

Ovarian structure and function is determined since the very first days of development when gonadal sex differentiation happens, through the stages of meiosis, follicle assembling and early recruitment, that happens in utero in humans (Puttabyatappa and Padmanabhan 2018). Humans are exposed to complex mixtures of environmental pollutants that can even cross the plancetal barrier (Mathiesen et al. 2021). About 800 of them have established hormone-disrupting properties hence so-called endocrine disrupting chemicals (EDCs)(Karthikeyan et al. 2021), such as bisphenol A (BPA), diethylhexylphthalate (DEHP), PFOA or PCB153 (Tamayo-Uria et al. 2019), whose exposure is continued during the entire lifespan (Hunt et al. 2016; Kahn et al. 2020). Some EDCs may also alter the normal function of adipose tissue and other metabolic organs like liver, shaping metabolically dysfunctional phenotypes and suspected of contributing to the development of obesity (Cano-Sancho et al. 2017a; Ren et al. 2020; Sun et al. 2022; Vrijheid et al. 2020). The

estrogenic plasticizer BPA is the unique chemical that has been systematically evaluated in relation to PCOS, causing the development if PCOS-like phenotypes in adult rats exposed during the developmental period (Fernández et al. 2010), disruption of ovarian steroidogenesis (Shi et al. 2021) and inconsistently associated with PCOS risk in the few small case-control studies available (Jala et al. 2022). Other EDCs have been seldom explored up to date, including phthalates (Vagi et al. 2014), organochloride insecticides (Guo et al. 2017; Vagi et al. 2014), PCBs (Yang et al. 2015) and per/polyfluorinated chemicals (Jala et al. 2022; Vagi et al. 2014).

A growing body of evidence suggest the capacity of some EDCs, known as obesogens, to activate signalling pathways related with metabolic dysfunction and obesity (Nadal et al. 2017). Obesity and insulin resistance are highly prevalent traits (50%-88%) among women with PCOS (Barber et al. 2019). The fact that pre-pubertal obesity has been prospectively associated with PCOS risk (Koivuaho et al. 2019) and hyperinsulinemia contributes to androgen-dependent anovulation through different mechanisms supports that PCOS may be a consequence of metabolic disruption (Sanchez-Garrido and Tena-Sempere 2020). For that reason, the mounting evidence showing the contribution of synthetic EDCs on metabolic dysfunction and childhood adiposity may help to explain the imbricated role of environment on PCOS pathogenesis (Cano-Sancho et al. 2017a; Heindel et al. 2015; Trasande et al. 2016). Noteworthy, there is a close relationship between signaling pathways activated by obesogenic chemicals and those related with the physiology of reproduction. This is the case of peroxisome proliferator-activated receptor gamma (PPARγ), prototypical receptor activated by a vast list of synthetic obesogens, and the alteration of steroidogenesis (Huang and Chen 2017).

Despite the existing evidence supporting the potential role of BPA, a prototypical EDC, on PCOS progression, many questions related the role of real-life chemical mixtures of EDCs still remain unanswered. Some methodological challenges may help to explain such a longstanding research gap:

Bottlenecks:

- *The physio-pathological framework relating the exposure to EDCs and development of PCOS remains unclear.* Understanding how prenatal and postnatal/adult environmental factors interplay along the natural history of a disease remains a key challenge (Clark et al. 2007; Puttabyatappa et al. 2016). Whereas a growing list of evidence support the pivotal role of prenatal exposure on metabolic and reproductive function programming, human epidemiological studies with such long follow-ups are extremely scarce. Noteworthy, all available observational studies on the links between EDCs and PCOS are small (n=30-171) clinical-based case-control designs (Jala et al. 2022), depicting the adult exposures at time of diagnosis, thus at high risk of reverse causation and exposure miss-classification. In addition, there is still little understanding how the EDC may interplay with the metabolic and endocrine disruption along the different manifestations of PCOS, such as early puberty related with prenatal exposures to EDCs by some studies (Uldbjerg et al. 2022).

- *Current studies on PCOS research lacked of comprehensive characterization of female chemical exposome.* In Europe more than 21,000 chemical compounds have been registered by European Chemicals Agency (ECHA), over 4700 of them produced above 100 tons per year. In addition 800 chemicals have been suspected of being hormone disruptors (Karthikeyan et al. 2021), however among them, only BPA have been comprehensively analysed in association with PCOS (Jala et al. 2022). None of the presently available studies have assessed the effect of mixtures (Jala et al. 2022), despite the evidence supporting the generalized exposure of women to complex chemical mixtures (Traoré et al. 2018). This is of paramount relevance because chemicals may interact on biological pathways, and thus failing to characterize the joint effects and interactions may result in null findings due to co-exposure bias (Lazarevic et al. 2019). The limited analytical capacity of instruments used to characterize the biomarkers of exposure may help to explain the fact that most epidemiological studies have been designed to target specific compounds or few congeners from the same family.

Novel cutting-edge high-resolution mass spectrometry are favoring a transformative process in biomonitoring methods, scaling-up the analytical capacity and molecular coverage with minimal biological sample requirements with automatable procedure to apply them in large epidemiological studies (Vermeulen et al. 2020; Walker et al. 2019).

- *Identification of causal relationships between chemical mixtures and health* remains a cornerstone in environmental research, traditionally achieved by a long iterative process integrating observational and controlled experimental studies (Whaley et al. 2016). The integration process can last for several decades with large economic and societal costs until regulatory agencies can develop evidence-based policies, thus not accommodated to the large amount of new chemicals released nowadays to the environment. Hence, the development of novel efficient strategies to support the causal chain of events in chemical risk assessment has been stressed to overcome the contemporary challenges of 21st Century, compliant with the "3R principles" to ensure reduction of animal experimentation. On this regard, the emergence of omic technologies such as metabolomics in epidemiological settings have shown large potential to gain insight on the mechanism underlying the statistical associations between exposures and apical disease phenotypes (Matta et al. 2022). The application of high-throughput technologies that generate high-amount data have the associated challenges of multiplicity, hence identifying the functional true links among highly noisy data. Considering the limitations of advanced statistics to overcome this issue, a new generation of methods driven by the biological knowledge has been stressed to gain, insight in a more statistically efficient and powered manner, on the functional interactions of the chemical exposome (Chung et al. 2021; Vermeulen 2022).

Hypothesis and main aim

The physiopathology and natural history of PCOS is not fully understood (Joham et al. 2022), hence the different events coexisting with PCOS timeline of events have not been completely delineated. The interplay of metabolic dysfunction and PCOS risk is specially intriguing, specially on the light that different studies have reported that alterations in adiposity precede the early manifestations of PCOS such as precocious puberty timing (O'Keeffe et al. 2020) and PCOS diagnosis (Koivuaho et al. 2019), thus suggesting that some causal link may be underlying instead of being a consequence. Considering the growing list of synthetic chemicals that are able of prenatally programming or modulate later in life the metabolic function, if have this project proposes the ***in utero*** **metabolic programming of polycystic ovary syndrome** as unifying hypothesis to understand the interplay between EDCs, metabolic dysfunction and PCOS risk and related early manifestations (Figure 30). Uncovering common metabolic fingerprints across the different life-stages of PCOS progression will provide novel clues to better understand the physiopathology and the identification of early biomarkers of the disease.

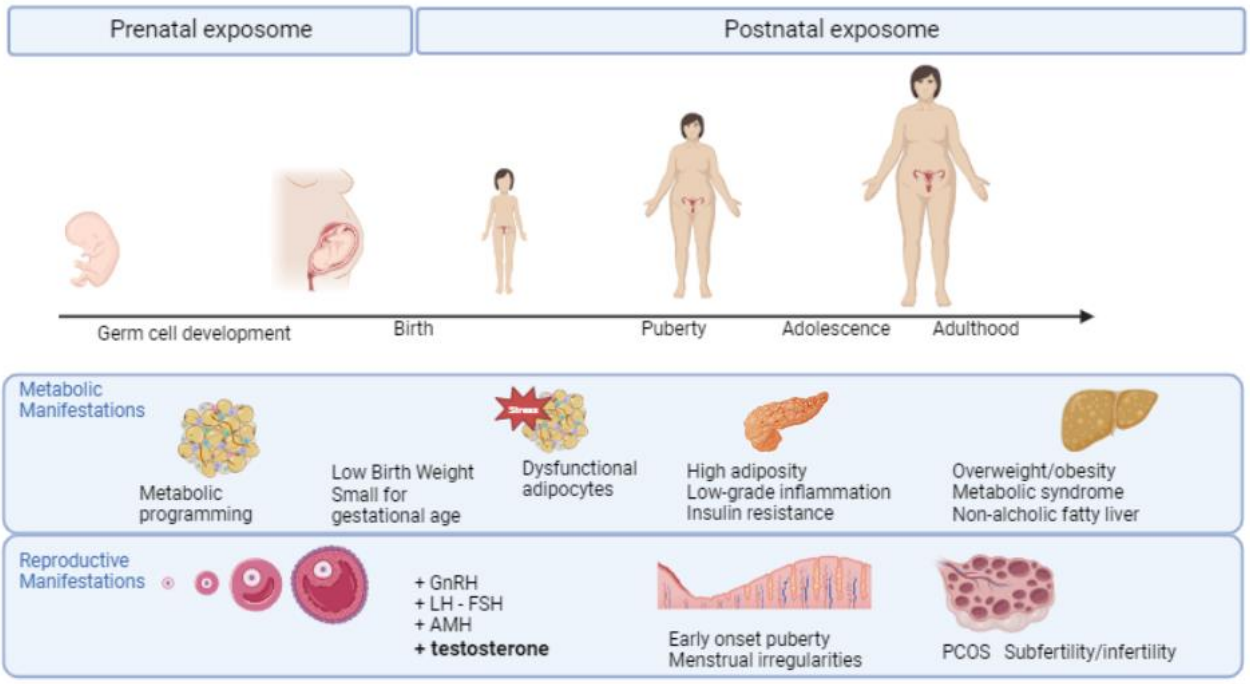


Figure 30. Representation of the metabolic programming of female reproductive health hypothesis

The **overarching aim** of the project is to establish the role of EDCs on PCOS risk since the prenatal period onwards, with special focus on the interplay of metabolic disruption as key linking mechanism to align early manifestations of hyperandrogenism such as early puberty and PCOS.

Systems Exposomics: Novel approach to a multifactorial problem

The EggXposome project embraces systems exposomics as a novel concept, integrating the principles of systems biology and exposomics, to circumvent current bottlenecks in environmental epidemiology and gain insight on the mechanistic relationships between environmental stressors, metabolic dysfunction and ovarian disorders (Figure 31). The novelty consists in an unique integrated approach specifically designed to characterize the chemical mixture risks of PCOS in women and metabolic markers informing of modes of action. *In silico* system-based models, *in vitro* high-throughput metabolomic assays and exposome-scale epidemiological studies will be integrated to characterize biologically active interactions involved in the physiopathology of PCOS. Multitargeted mass-spectrometry methods will be specifically developed to characterize the PCOS exposome-metabolome interactions driven by system biology and assisted by advanced machine learning algorithms. The results of this project will provide essential information on the internal chemical exposome of women and its role on common reproductive disorders with large societal and economic costs. Chemical risk assessment and intervention strategies will be modelled to support regulatory and policy decision-making to safeguard female reproductive health.

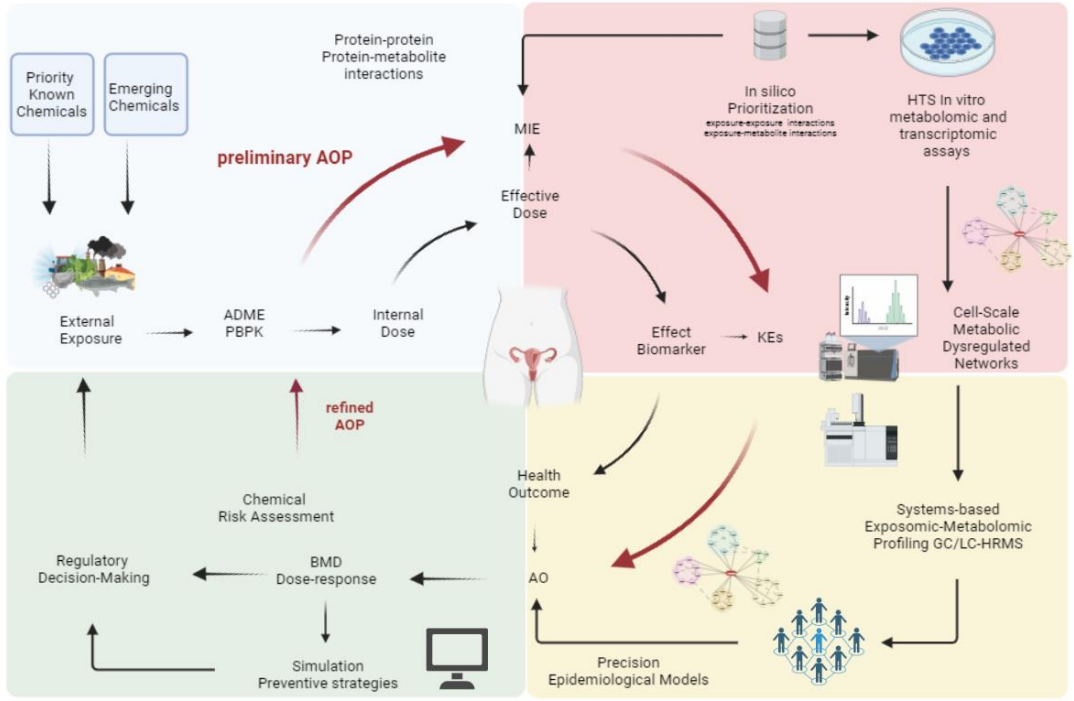


Figure 31. Novel methodological framework proposed in the present study

Feasibility

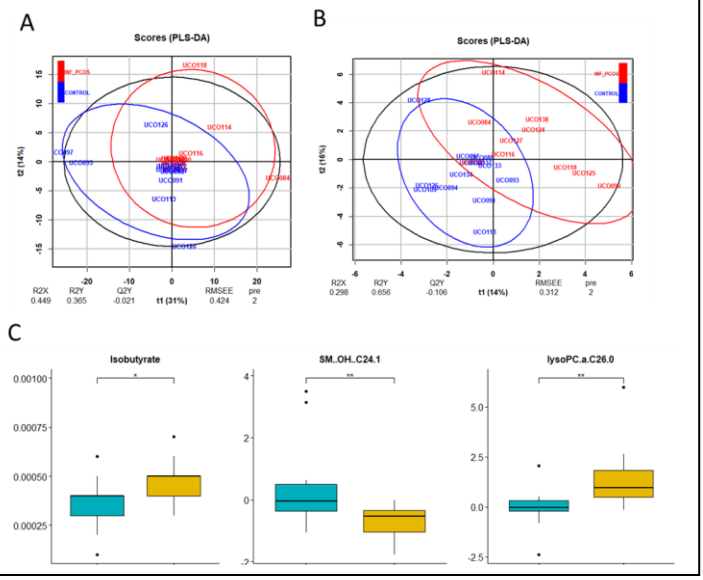
The proposed hypothesis and concept are built on my multidisciplinary expertise developed around nutrition, the toxicology of EDCs on metabolic function, epidemiological modelling of chemical mixtures and the metabolic perturbations related to the toxicity of EDC on female reproductive health.

During the last years, I have focused my research on developing novel integrative approaches based on metabolomics and exposomic profiling to gain insight on the causal associations between EDCs and female reproductive diseases in observational studies. To this end, I have conceived a first proof-of-concept study on the relationships between persistent organic pollutants (POPs) and endometriosis risk, showing the discovery potential either to identify novel clues on the relationships between POPs and the disease, and potential biomarkers that could be used in clinical research to support non-invasive predictive diagnostic tools (Matta et al. 2022). I have extended the research to gain insight on the relationship between chemical mixtures of POPs, ovarian function and *in vitro* fertilization (IVF) outcomes among women undergoing Assisted Reproductive Technology (ART). The results showed that women are widely exposed to mixtures to POPs and suggested that some of them, like trans-nonachlor and PCB189, negatively related with markers of ovarian function like antral follicle count (AFC) and AMH, especially among smokers (Lefebvre et al. 2022a; Lefebvre et al. 2022b).

I have also conducted a pilot study with the 10 women attending ART with infertility related to PCOS and 13 women identified as controls with similar age and body mass index. A comprehensive characterization of metabolic fingerprints by high-resolution mass-spectrometry approaches (HRMS) and nuclear magnetic resonance (NMR) was conducted, resulting in over 700 quantified compounds. Despite the small sample size, preliminary statistical analysis have shown that women with PCOS have a specific metabolic fingerprint from HRMS (Figure 32A) and NMR (Figure 32B) technologies. The most discriminative metabolites were isobutyrate (NMR) and sphingomyelin (OH) C24.1 (MS) and lyso phosphatidylcholine a C26.0 (MS), as shown in Figure 32C. Whereas these findings are not conclusive, they support the proposed hypothesis on dysmetabolic

programmation, and consistent with previous studies where high levels of isobutyrate have been also found among women with diminished ovarian reserve and dysbiosis (Li et al. 2022).

Figure 32. Metabolic profiles by LCMS/MS (A) and NMR (B) of women with and without PCOS attending IVF, including the most discriminant metabolites (C). Preliminary unpublished data from EndoxOmics-β projet extended to PCOS.



I had also the opportunity to develop some specific *in vitro* research with un-differentiated and mature adipocytes, and follow-up their metabolic behaviour after being exposed to non-toxic doses of synthetic chemicals assumed to safe like triphenyl phosphate (Cano-Sancho et al. 2017b) or parabens (Elmore et al. 2020), among others. The result from that experience supported the fact that adipose tissue is a sensitive organ to EDCs, vulnerable to the adipogenesis programming or the development of metabolically dysfunctional phenotypes. My epidemiological research has also supported the fact that prenatal exposure to EDCs can be associated with later childhood adiposity markers or cardiometabolic risk (Cano-Sancho et al. 2017a; Güil-Oumrait et al. 2022). One of the major complexities in epidemiological research with environmental pollutants is related to the characterization of all causally related compounds that if not measured, can prone to bias amplification (Weisskopf et al. 2018) or co-exposure confounding (Cano-Sancho and Casas 2021). To address such problem, first there is the need of developing comprehensive exposure assessment methods covering the most relevant compounds related to the disease. A new generation of HRMS has shown the high potential to extend the coverage of organic exposure biomarkers through targeted or non-targeted ways from tens to thousands, hence providing a unique opportunity for the chemical exposome research. In a proof-of-concept study conducted with breast milk, I have combined ultra-trace multitargeted and non-targeted methods in order to characterize the chemical exposome of milk, resulting in more than 800 chemical features from nutritional, microbial and toxicant compounds, some of them related with pre-term infant growth stepping up the chemical coverage of previous methods (Cano-Sancho et al. 2020a). Since then, other novel multi-targeted methods based on HRMS have been proposed to gain insight on the chemical exposome with quantitative resolution and extensive coverage, overcoming the complexity of combining multiple analytical platforms with minimal sample volume. For instance scalable frameworks to screen 1922 lipids (Medina et al. 2022) or over 1019 chemical species (e.g. steroids, endocrine disruptors, pharmaceuticals, microbiota metabolites) in 200 □ L of serum (González-Domínguez et al. 2020). These studies support the large analytical capacity of novel HRMS instruments to gain insight on the chemical complexity of internal exposome and the potential application in large epidemiological studies, offering quantitative measurements, unique chemical identity, large coverage and scalability by automation.

A major statistical challenge for an efficient use of high-throughput platforms in epidemiological research is dealing with multiplicity (identifying the true associations among a large number of potential candidates). This is specially challenging with metabolomics and exposomic data which, conversely to genomic data, are characterized by large intra-individual variability and measurement error. For that reason, there is growing interest on informing with prior biological information to

inform the statistical analysis (Vermeulen 2022). By integrating a priori biological knowledge about probable mechanisms and signalling pathways becomes a way efficiently implemented in other biological fields such as genomics and metabolomics (Rosato et al. 2018). Noteworthy, the biological *priors* are often a prerequisite to design the analytical strategies (e.g. selection of priority biomarkers), however up to date there are no strategies to unify the potential of systems biology to inform the development of analytical strategies targeting specific biological pathways and the related interactome. Within the framework of an ongoing project (EndoxOmics), I am developing a prioritization framework focused to endometriosis, integrating the principles of adverse outcome pathways and systems biology to guide text-mining algorithms through structured ontologies (Delmas et al. 2021), to favour the ranking of priority toxicants and endogenous metabolites related with molecular pathways related to endometriosis, a basis model to extend in the case study of PCOS.

Finally, elucidating complex associations between environmental chemicals and health outcomes remains a key challenge leveraged by a list of advanced statistical solutions to characterize the joint effect of compounds, non-linear associations or interactions between pollutants or between pollutants and other bioactive/endogenous compounds. During a recent collaboration conducted with ISGlobal in the framework of ATHLETE and OBERON H2020 projects, I was able to prove the interest of applying a comprehensive machine leaning framework to identify novel pollutant-pollutant and pollutant-nutrient interactions during pregnancy on the associations with child obesity . That study provided further evidence about the high potential of combining advanced statistical methods such as gradient boosting machines or Bayesian Kernel machine regression to characterize complex data-patterns and favouring the identification of high-risk sub-populations that otherwise would not be identified. The underlying issue reflects the need of moving epidemiological research from whole populations to individuals or group of them, as manifested with the principle of precision public health (Velmovitsky et al. 2021).

Globally, the complex project aim demands the integration of multiple disciplines and skills articulated through advanced translational methodologies. My multi-skilled profile with experience on the main domains involved in the project will ensure the efficient development of novel integrative methods, supported by specialists in each involved discipline to ensure the specific training on novel approaches integrated in the project such as systems toxicology. Thus, I will ensure the implementation of the novel framework by consolidating a team of postdoctoral and predoctoral researchers and research assistants specifically trained to achieve the different project goals. Despite my experience on *in vitro* models, I have limited competences on systems toxicology and *in vitro* metabolomics, hence a specific training of the team will be ensured by the specialists Dr. Karine Audouze and Prof. Xavier Coumoul (INSERM). There may appear some constraints when setting up the proposed novel multi-targeted exposome-metabolome analytical methodology ambitioning to cover over 2000 endogenous and exogenous chemicals. In such case, conventional methodologies that I am currently using will ensure the characterization of about 1000 chemicals using commercial solutions for quantitative metabolomics such as Biocrates MxP500. The application of novel methods in existing cohorts with approved ethical consents will minimize the delays to access to the data or the biocollections. The lack of statistical power may impair the sensibility to identify mildest associations, for that reason the integration of a priori biological knowledge and a comprehensive study or temporal variability or exposome-metabolome interactome will contribute to develop more statistically efficient strategies, in addition of a comprehensive environment-wide analysis conducted in the large Nutrient-Santé cohort. Finally, I have also limited experience on functional analysis, thus I will expand the competences of the team with external training in the topic with leader researchers such as Dr. N. Poupin (INRAE) to support the development of novel methods.

Innovation potential and expected impacts

The current research linking environmental chemical stressors with PCOS is very scarce, mostly focused on few individual chemicals and conducted in small case-control studies conducted in adult females, neglecting the effect of chemical mixtures. The present project proposes **a ground-breaking approach to accommodate exposome-scale exposure assessment methods in epidemiological studies** in order to extend the coverage of chemical compounds within the range of hundreds of EDCs and over 1000 endogenous metabolites (global metabolome and steroidome) to gain insight on the potential linking mechanisms. The integration of *prior* biological knowledge in the pipeline using system-based methods, is another novelty to overcome the limits of fully data-driven methods that characterize nowadays the epidemiological research. I will also conduct a comprehensive analysis of data from NutrinetSante cohort, one of the largest ongoing cohort studies with comprehensive data on dietary exposures (500 food contaminants), dietary patterns, lifestyle and environment including over 62,714 women, among which we will confirm roughly 3000 cases of PCOS. Noteworthy, the integrated *in silico* and *in vitro* framework with expo-metabolomic epidemiological settings has the ambition of revealing underlying modes of action linking individual EDCs and their mixtures, with the life-course of PCOS.

The proposed project has the ambition of substantially contributing to fill the **longstanding fundamental gaps on the role of EDCs in the physiopathology of PCOS**. The integrative approach will boost the identification of prenatal, childhood or adulthood chemical risk factors to support the design of preventive strategies. The integration of systems biology and metabolomic analysis will help to decipher the underlying mechanisms and mode of actions linking EDCs and PCOS. The integration of a birth-cohort with adult cohorts will provide the unique opportunity of developing life-course models of disease progression. The findings will also contribute to better understand the crosstalk between metabolic dysfunction and PCOS risk, with the large potential of identifying putative predictive biomarkers to support the early diagnosis and/or prognosis.

The findings will contribute to the development of **evidence-based of policies of non-communicable disease (NCDs) prevention** at national and European level, aligend with the European Strategy for the Prevention and Control of NCDs or the implementation of World Health Organization 2030 Action Plan for Sexual and Reproductive Health. The project design has the ambition of identifying main drivers of risk, but also dietary and lifestyle modulators and determinants in order to provide actionable recommendations to design effective prevention strategies to reduce the exposure burden and disease risk at diet and food system level.

The novel methodological framework will help to push the exposome concept towards a **next generation strategies in chemical risk assessment** (Vermeulen et al. 2020), favoring the implementation of high-throughput and efficient approaches to assist the rapid safety evaluations and compliant with the reduction of animal experimentation ("3R"). The integration of omic-based *in vitro* studies with exposomic-scale epidemiological approaches will allow the efficient translation of basic research finding in operational information for regulatory decision-making, contributing to the ongoing transformative process embraced by major regulatory agencies worldwide (Hartung and Tsatsakis 2021; Sillé et al. 2020). The resources generated by *in vitro* and *in silico* studies will favor the development of predictive models and read-across to support animal-free chemical risk assessment.

Methodology

In order to fulfil the scientific gaps underlying the potential role of exposure to mixtures of EDCs on PCOS risk, I will constitute a multidisciplinary team to integrate state-of-the-art *in silico* and *in vitro* metabolomics methods (WP1), with the most advanced HRMS technology (WP2) and statistical methods to profile the chemical space of PCOS in exposome-scale epidemiological studies (WP3,4) and favour the reconstruction of potential modes of action to develop risk assessment and prevention strategies (WP5).

First, we will systematically structure the available evidence linking EDCs and the different manifestations of hyperandrogenism such as early puberty and PCOS, scaffolding a preliminary AOP (Matta et al. 2021). We will leverage curated data from currently available resources like Comparative Toxicogenomics Database (Chai et al. 2021; Davis et al. 2019), peer-reviewed literature available and specific resources such as AOPwiki, following according the Organisation for Economic Co-operation and Development guidelines [41] and assisted by text-mining tools such as AOP-helpFinder (Carvaillo et al. 2019; Jornod et al. 2021). **Systems-based computational models** applied in toxicological databases such as ToxCast and Comparative Toxicogenomic Databases will be used to map the biological pathways potentially altered by EDCs based on protein-protein association network (PPAN) analysis (Audouze et al. 2013; Wu et al. 2020) and extended with the protein-metabolite interactome leveraging publicly available protein-metabolite interaction database ConsensusPathDB (Kamburov and Herwig 2022). A **cell-based metabolomics screening** will be conducted in a subset of 30 priority chemicals based on the outputs systems-based prioritization (Malinowska et al. 2022), with the aim of characterize the metabolic dysregulations at a molecular and cellular level, support the structural characterization of metabolic by-products and the identification of modes of action.

The outcomes from *in silico* and *in vitro* models will allow the identification of main biological pathways dysregulated in the physiopathology of PCOS, likewise the inventory of endogenous and exogenous compounds likely to interact with them to favour the development of tailored biomarker profiling methods. On this regard, a novel analytical strategy will be developed to conduct a comprehensive characterization of the chemical exposome of female in serum and urine specifically designed to cover the PCOS exposome-metabolome-steroidome interfaces. **The analytical strategy will be built on existing state-of-the-art multi-targeted methods** developed to minimize the amount of biological sample, ensure the scalability for application in large cohorts and the ensuring the quantification, prerequisites for application in large epidemiological studies. Hydrophobic toxicants including organohalogenated EDC (PCBs, pesticides, per/polyfluorinated chemicals) will be characterized in serum with gas chromatography (GC)-HRMS optimized to low sample volumes and scalable 96-well plate method as described in the literature (Stubleski et al. 2018). A global quantitative method to analyse the lipidome will be applied in serum using couple to hydrophilic interaction liquid chromatography MS tandem covering over 900 compounds from the main chemical families (sphingolipids, phosphatidylcholines, acylcarnitines, etc) as described recently (Medina et al. 2022). A multi-target quantitative and automatable method based on liquid chromatography (LC) HRMS covering over 1000 endogenous and exogenous chemical compounds will be set up based on a presently developed method (González-Domínguez et al. 2020), to complete the profiling of more hydrophilic compounds in serum and urine, including steroids, non-persistent pesticides, bisphenols, phthalates, perfluorinated chemicals, bile acids, microbiota metabolite, dietary metabolites, short-chain metabolites, phamaceuticals, polyphenolic compounds, and mycotoxins.

The biological interpretation of biomarkers directly relates with its stability over the time and the reliability to depict a specific underlying metabolic state (or exposure level) (Agueusop et al. 2020). Hence, in order to develop a robust epidemiological approach based on single time points we will first conduct a time course study to characterize the stability of biomarkers, the dynamics of exposome-metabolome interactions and the identification of determinants of intraindividual, temporal and seasonal variability. To this end, urine and blood samples from women that participated in the longitudinal MEDINET cohort (University of Liege, Belgium 2022-2023) will be characterized using the novel exposome-metabolome. In this unique time-course study, 16 women at childbearing age were followed-up during a year, weekly for the first 10 weeks, and monthly over the rest of the study collecting blood, urine and saliva each time point in addition of administering a lifestyle questionnaire.

The relationships between the adult exposure to priority EDCs and PCOS risk will be characterized in **the French large-population based cohort Nutrinet-Santé** including nowadays 174,205 participants with an overrepresentation of women (~72%) and a median follow-up between 2009-2021 of 7.8 years (Hercberg et al. 2010). The cases of PCOS (n~3000) will be validated by specialized gynaecologist by inspection of the clinical histories. Among the sub-group of women that participated in a biocollection between 2011 and 2014, a nested case-control study with 106 confirmed cases of PCOS will be established to apply the novel exposomic-metabolomic profiling method. Statistical analysis will be conducted to identify environmental and dietary factors associated with PCOS risk applied 1) to the full cohort of women using dietary exposure modelling methods integrating data for over 500 food chemicals and contaminants (Baudry et al. 2021; Chazelas et al. 2021; Fleury et al. 2017) and 2) the nested case-control subpopulation using the data generated with omic-scale biomarker-based profiling method. The associations between prenatal exposure to EDCs and aberrant puberty timing, as a hallmark of early manifestations of hyperandrogenism, will be characterized in the **European-wide HELIX birth cohort** (Maitre et al. 2018; Vrijheid et al. 2020) involving 521 mother-daughter pairs. Advanced statistical models like the Bayesian Kernel Machine Regression will be used to characterize the effect chemical mixture, and high-dimensional models will be used to identify the potential role of metabolic dysfunction, leveraging available exposome and metabolome-wide datasets (Papadopoulou et al. 2019).

A comprehensive pathway analysis and metabolic network overlapping study will be conducted to identify common **molecular pathways and modes of action** to gains insight on the physiopathology of environmental hyperandrogenism, support the chemical risk assessment and the design of prevention strategies. Translating the fundamental findings into actionable public health policies and useful information for regulatory decision-making a final set of actions will developed focused on 1) functional/pathway analysis; 2) developing a novel framework to translate metabolomics data into common regulatory decision-making frameworks (defining mechanistic points of departure based on benchmark dose modelling) and 3) identify potential interventions at diet and food chain level to reduce the exposure of chemicals or counterbalance the effects.

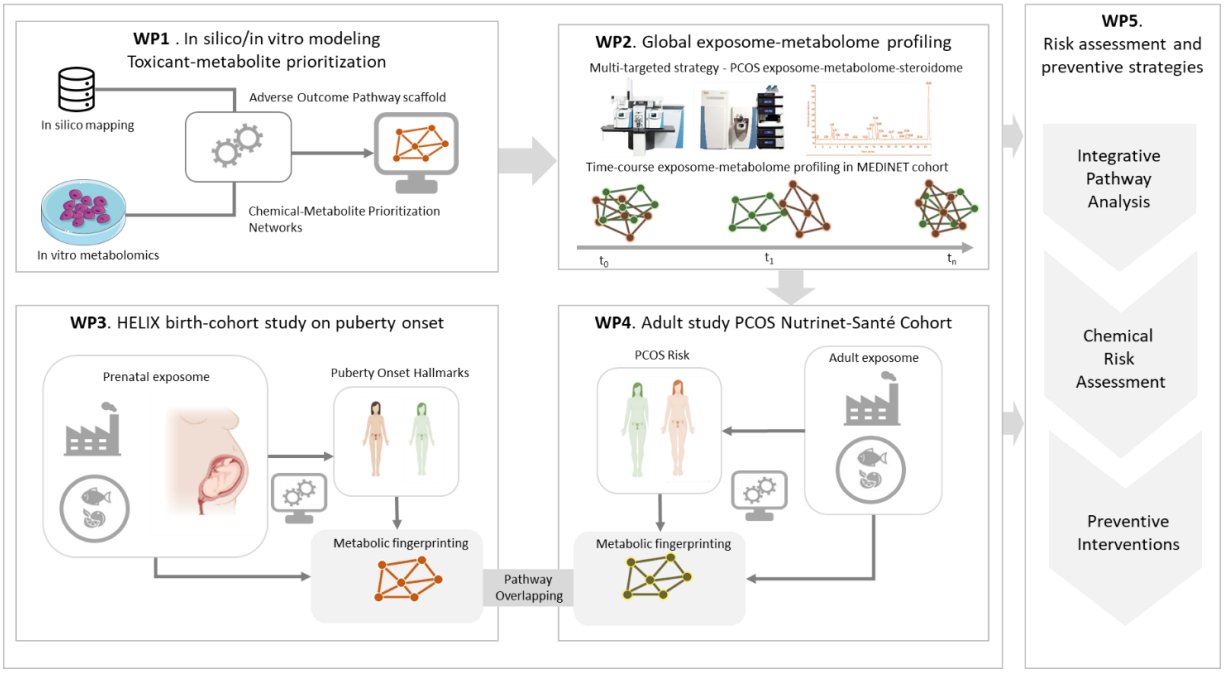


Figure 33. Structuration of the project and interaction between work-packages

4. CANDIDATE AND SCIENTIFIC PRODUCTION

4.1 Presentation of hosting institution and positioning

Eleven years after the creation of the National Veterinary School of Nantes (1979), the Laboratoire des Dosages Hormonaux becomes the LDH / LNR after being appointed by the Ministry of Agriculture as National Reference Laboratory for the surveillance and control of growth promoters in animal and animal products.

- In 1995, the structure was for the first time in its history accredited by COFRAC according to the EN 45001 standard, and later on to the new version of this standard (ISO17025).
- In 2000, the laboratory becomes National Reference Laboratory for the control of dioxins and changes its name in order to integrate environmental contaminants and better stick to its activities. It becomes LABERCA.
- In 2001, the unit sets up an international training for third countries targeting the control systems for residues and contaminants in food; the acronym given to this training is SARAF for School for Advanced Residue Analysis in Food.
- In 2003, LABERCA certifies its Research activities according to the ISO9001 standard. Several years later, SARAF is also ISO9001 certified, followed by the analytical chemistry platform.
- It celebrates its 100th rank A scientific publication in international peer-reviewed journals in 2007. In the meantime, LABERCA's NRL activity has expanded its scope by first hooking polycyclic aromatic hydrocarbons, then melamine and finally non dioxin-like polychlorinated biphenyls to its reference mandate.
- As of 2009, the laboratory workforce is growing significantly; the structure is better equipped to enter the major European research projects (FP6, FP7) and to invest in large national studies first alongside AFSSA (Anses) and then InVS (SPF). The unit will pass the evaluations of its activities by the AERES then by the HCERES by hanging respectively an A + and an excellent mention for its Research. The next evaluation is planned for the end of 2020.
- In 2015, the laboratory gets accredited for the organisation of inter-laboratory tests according to ISO 17043.
- In 2017, LABERCA becomes Joint Research Unit Oniris-INRAE, ALIM-H department and enters the European project H2020 HBM4EU.
- In 2018, LABERCA integrates the coordination unit of the newly created food chain monitoring platform (PF SCA), coordinated by the DGAL.
- In 2019, the structure celebrates its 400th publication in international scientific journals.

Research activities

The laboratory's research activity is framed on the one hand by quadrennial contracts entered into with its two "Research" supervisions, which are the AlimH department of INRAE and the DGER, but also with its DGAl supervision on the basis of an annual agreement. The LABERCA builds its projects with regard to the strategic axes of the INRAE Orientation documents, ensuring that they fit within its thematic scope but also that they are in direct connection when possible with its NRL missions such as described by regulatory texts.

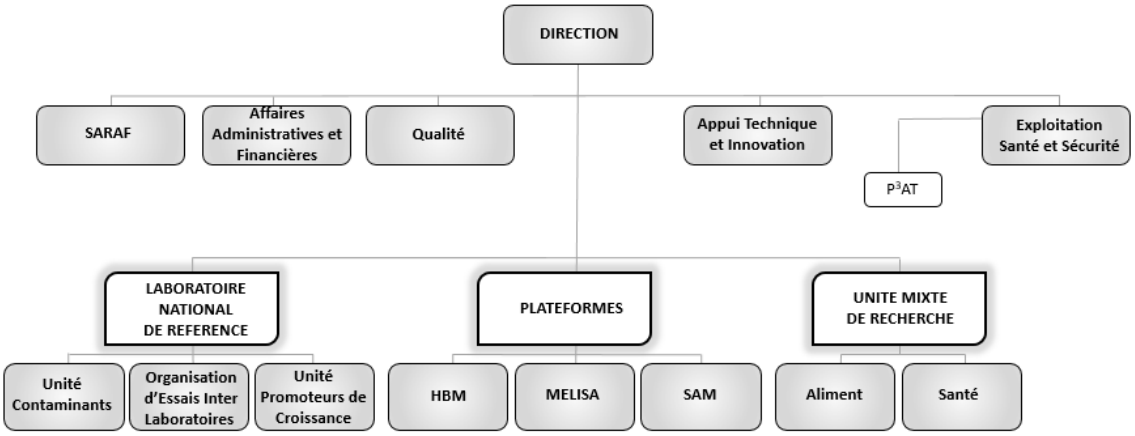


Figure 34. Organization chart of LABERCA

From a scientific point of view, the Unit's historical activity area is that of chemical food safety and is part of a global and integrated approach to the characterization of exposures, from agro-supply to humans and their descendants. LABERCA is committed to generating data and knowledge to characterize both the external exposure (food) of the consumer (measures of occurrence in food) but also its internal exposure (indicator of impregnation) with regard to the same parameters, their metabolites or their degradation products in biological fluids and tissues. The Unit also generates data that contributes to hazard identification, particularly for endocrine disrupting substances, by identifying biomarkers of effect (metabolome, lipidome or steroidome). The Unit also contributes to the study of possible associations between exposure markers (external or internal), effect markers (omic disruptions) and clinical observations, especially in the field of reproduction and development. The project of the Unit aims to perfect the knowledge around the notion of exposome in all its complexity, especially with regard to emerging chemical contaminants.

The laboratory's research activity is framed on the one hand by quadrennial contracts entered into with its two "Research" supervisions, which are the AlimH department of INRAE and the DGER, but also with its DGAl supervision on the basis of an annual agreement. The LABERCA builds its projects with regard to the strategic axes of the INRAE Orientation documents, ensuring that they fit within its thematic scope but also that they are in direct connection when possible with its NRL missions such as described by regulatory texts.

The structure of LABERCA's research activities is based on an organization by project management, from conception to project promotion; LABERCA is certified as such according to the ISO9001: 2015 standard. The matrix organization of research is defined by a structure made up of 2 main themes, one "Food", the other "Health" and two associated Scientific Officers who form the Scientific Directorate. Research funding is provided by the budgets allocated by the DGAl (annual agreement), and upon response to calls for tenders in its area of competence by the European Commission (contract), the ANR (contract), ANSES (contract), and Regions (contract), for the main.

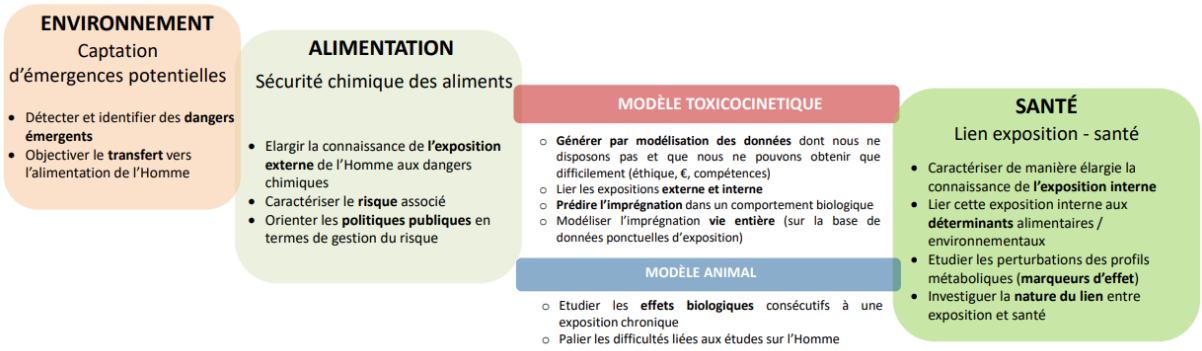


Figure 35. Structure of research topics developed for the period 2021-2025.

I joined the LABERCA in October 2016 to contribute on the research aims identified within the Health axis, notably to:

- Identify and address the main challenges and limitations associated to the most currently used biomarkers of exposure.
- Application of novel methodological strategies to identify associations between existing and/or newly constructed biomarkers of exposure and health outcomes in a complex mixture / co-exposure context.

4.2 Presentation of candidate

4.2.1 Curriculum Vitae

Personal Information

Family name, First name: CANO-SANCHO, German (Male, married)
Date of birth: 08 November 1981
Nationality: Spanish
Researcher unique identifier (s) (ORCID): 0000-0003-4111-4007
National (Spain) Identification Number (DNI/NIF): 43748736E

Education

2013 PhD on Food an Agricultural Science and Technology, University of Lleida, Lleida, Spain (Cum Laude). Dissertation: Exposure assessment of Catalonian population to mycotoxins. PhD supervisors: Prof. Vicent Sanchis, Dr. Sonia Marin.

2010 Master's Degree in Research on Agro-food Production Systems, University of Lleida, Lleida, Spain, 2010.

2008 Degree in Food Science and Technology, University of Lleida, Lleida, Spain, 2008.

2006 Bachelor in Human Nutrition and Dietetics, University Rovira I Virgili, Reus, Spain, 2006.

Fellowships, scholarships and awards

2009 University of Lleida Competitive Pre-doctoral research fellowship

2010 Agency for Management of University and Research Grants (AGAUR) Pre-doctoral scholarship

2015 Doctoral Thesis Extraordinary Award, University of Lleida, Spain

2019 Prix Etoiles Montantes de Recherche 2019, Région Pays de la Loire

Professional Experience

Current position

2016- Permanent Scientist INRAe (Chargé de Recherche, CR) LABERCA – UMR1329 ONIRIS, INRAe, Nantes, France.

Previous positions

2015-2016 Postdoctoral researcher University of California at Davis, Environmental Toxicology Department, Davis CA, United States. Supervisor: Prof Michelle La Merrill (UC Davis), Prof Andrew Salmon (EPA, OEHHA)

2013-2014 Postdoctoral researcher University Rovira I Virgili, Center of Environmental Technology, Food and Toxicology, Tarragona, Spain. Supervisor: Prof. Jose Luis Domingo, Dr. Marti Nadal

2008-2013 PhD student University of Lleida, Food Technology Department, Lleida, Spain. Supervisor: Prof. Vicente Sanchis, Prof. Sonia Marin

Visiting Scholars

2012 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) Departamento de Medio Ambiente, Madrid, SPAIN.

2010 TOXALIM UMR 1331 INRA/INP/UPS, Toulouse. Avisor: Dr. Isabelle Oswald.

2010 MATHEMATICS AND APPLIED INFORMATICS UNIT Institut de National de Recherche Agronomique (INRA) - Centre de Jouy en Josas, Advisor: Dr. Jean-Pierre Gauchi.

Languages

Spanish (maternal), Catalan, English and French

4.2.2 Teaching activities

Undergraduate and Master Student Supervision (n=13)

2021 Master 2 Reprodev (Reproduction et développement) Université De Paris – Universite Paris Sud. Metabolomic profiles of women with endometriosis and infertility: influence of exposure to chemical pollutants. (M. Gauthier DUVAL).

2021 Master 2, University of Lleida. Food and agricultural research. Exposure to Catalonian population to mycotoxins (Jose-Antonio GALLARDO).

2020 Master 2 Modélisation statistique. Faculté des sciences et techniques de Besançon. Application des méthodes multi-blocs pour l'intégration de données exposomiques et métabolomiques en études de santé environnementale (Nissia KAOUANE).

2019 Degree Project. Faculté des sciences et techniques de Besançon. Déterminants des coefficients de partage des polluants organiques persistants entre le tissu adipeux et le sérum : une approche basée sur des algorithmes d'apprentissage statistique (Nissia KAOUANE).

2018 Master 2 Sante Publique Et Risques Environnementaux. Universite Paris Descartes, Universite Paris Sud, Universite De Lorraine, Ecole Des Hautes Etudes De Sante Publique (EHESP). Application De Methodes D'apprentissage Statistique Pour Évaluer L'association Entre L'exposition Aux Polluants Chimiques Et L'endométriose (Delphine Mouret)

2017 Degree Project Pharmacie (DFASP2) filière industrie, Université Paris-Sud. Développement de nouveaux marqueurs de bio-surveillance pour étudier l'association entre l'exposition interne aux polluants chimiques et la santé : étude de cas sur les polluants organiques persistants et l'endométriose Léa LABRUNE

2015 Master on Environmental Toxicology, Univesity of California at Davis, US. In vitro approach to assess the obesogenic effects of parabens. Anna Smith

2015 Master on Environmental Toxicology, Univesity of California at Davis, US. In vitro approach to assess the obesogenic effects of DDT. Tansy Gu

2012 Degree Project on Agricultural Engineering 'Efecto del procesado del pan y la pasta sobre deoxinivalenol, deoxinivalenol-3- glucósido y zearalenona e importancia del efecto de la cocción de la pasta sobre la evaluación de la exposición a deoxinivalenol de la población catalana. Center: University of Lleida

2011 Degree Project on Agricultural Engineering ' Estudi del ratio esfinganina: esfingosina (Sa:So) en orina com a biomarcador d'exposició humana a fumonisines'. Center: University of Lleida

2011 Degree Project of Agrofood Industries Engineering 'Estimació de la ingesta de fumonisines, zearalenona i patulina per part de tres grups de població especials: gent gran, immigrants I celíacs.' Center: University of Lleida

2010 Bachelor Project of Agrofood Industries Engineering 'Análisis de micotoxinas en alimentos aptos para celíacos y alimentos consumidos habitualmente entre la población inmigrante.' Center: University of Lleida

2010 Bachelor Project of Agrofood Industries Engineering 'Exposición de la población catalana a la micotoxina fumonisima al consumir productos basados en cereales, principalmente maiz y cebada.' Center: University of Lleida

PhD Student Supervision (n=4, 2 defended)

2021-2024 Jose Antonio Gallardo (2021-2024). "Identificación y caracterización de la exposición a micotoxinas: co-ocurrencia y co-exposición con otros peligros químicos" Role: Co-supervisor with Prof. V. Sanchis. University of Lleida, Spain. Funding: Agency for Management of University and Research Grants from Catalonia, Spain.

2021-2023 Etienne BABIN (2021-2024). « Integration and analysis of exposomic and metabolomic data in observational studies: application to the link between chemical exposure and human health » Role: Co-supervisor with JP ANTIGNAC, E VIGNEAU; Funding: Co-Financement Région Pays de la Loire-INRAE).

2020-2022 Tiphaine LEFEBVRE. « Etude du lien entre l'exposition aux polluants organiques persistants (POPs) et l'infertilite chez la femme » Role : Co-supervisor with JP ANTIGNAC and S. PLOTEAU. Funding : self-funded CHU / Région Pays de la Loire Etoiles Montantes 2019. Defense date: 10/2022

2019-2021 Komodo MATTA. "Study of the associations between exposure to endocrine disrupting chemicals and endometriosis: an integrative approach" Role : Co-supervisor with JP ANTIGNAC and S. PLOTEAU. Funding: Allocation doctorale Co-financement ONIRIS- Région Pays de la Loire CPER2019. Région Pays de la Loire Etoiles Montantes 2019. Defense date: 2/7/2021. Currently postdoc at IARC, Lyon.

Other teaching activities

Member of Thesis Jury (n=4)

2023 I, REINA PÉREZ. Total effective obesogenic burden: new biomarker of combined effect to obesogens as a risk factor in obesity. University of Granada, Granada, Spain.

2020 X, PASCARI. Fate of Fusarium mycotoxins during food production. Cases of beer and cereal–based infant food University of Lleida, Spain

2019 E, ADOAMNEI. Determinants of reproductive parameters in healthy university males. University of Murcia, Spain.

2018 L, ALDARS-GARCÍA. Predictive mycology as a tool for controlling and preventing the aflatoxin risk in postharvest University of Lleida, Spain.

Member of Doctoral Committee / Comité de suivi inviduelle (CSI) (n=3)

2023-2025 M. GHOZAL (CRESS-UMR1153 Equipe EAROH) Exposition aux contaminants alimentaires en période périnatale et allergies pendant l'enfance. Supervisors : Blandine de Lauzon-Guillain.

2020-2023 L. FRESNAIS (ToxAlim, INRAE) Développement de nouvelles approches de biologie systémique pour l'évaluation de la toxicité de matières premières cosmétiques. Supervisors : Jourdan Fabien, Nathalie Poupin, Olivier Perin

2020-2023 N. GUIL (IS Global, Spain). Supervisors : Maribel Casas, Martine Vrijheid

Invited Seminars

2022 Multiexposure to chemical hazards. Master on Food Safety. University of Lleida.

2021 "An applied framework for statistical analysis of interactions in environmental-health studies" ISGlobal, Exposome Hub – WG: Advanced data modelling for exposure mixtures and causal inference.

4.2.3 Scientific societies

2021- Member of Scientific Committee of French "Fondation pour la Recherche sur l'Endométriose"

2021- Member of the International Society for Environmental Epidemiology

2019- Member of Grading of Recommendations Assessment, Development, and Evaluation (GRADE) Working on Environmental Health

4.2.4 List of research projects and funding grant proposals

Current Projects

2023-2026. CHEMVAX. Effets de l'exposition aux substances perfluorées alkylées sur les réponses vaccinales au Covid 19 chez l'homme. Partners: Inserm UMRS1124 (PI: Etienne Blanc) ANSES2023 projet n°2023/EST/136 (160k€) [Role : WP leader]

2022-2025. EXPOL. Approche Exposome pour l'identification de causes environnementales du Cancer du Poumon chez les femmes non-fumeuses. Partners: Inserm U1018 (PI: Loredana Radoi) ANSES2022 projet n°2022/EST/210 (200k€) [Role : Partner]

2023-2027. Exposition aux polluants organiques persistants et sévérité de l'endométriose dans la cohorte ComPaRe-Endométriose. Partners: Inserm U1018 (PI: Marina Kvaskoff) Agence Nationale de la Recherche 2024 (684 k€). [Role : WP leader]

2022-2024. EndoxOmics. Persistent organic pollutants and endometriosis: a metabolome wide association study. French Medical Research Foundation ('Fondation pour la Recherche Médicale'). Partners: Inserm U1018 and INRAE ToxAlim. 518k eur [Principal investigator]

2022-2027. PARC. Partnership for the Assessment of Risks from Chemicals. European Union Horizon 2021-2027. WP4.1 and WP6.2 [Partner]

2022-2024. BCEXPOS, AAP CGO2021 – Project Collaboratif 320 k eur . Leader IRSET (V. Bessonneau)

2021-2023. LactOMICs. AAP MG SYALSA2021 INRAE Units: PhAN, Stat-SC, SECALIM, CRESS-EREN. Non-INRAE Units: MERIT, CHU Nantes.[Co-Principal Investigator]

2019-2022. GAMMA. Leader Institut Gustave-Roussy. AAP Fondation France 210 k eur [Partner]

Selection of Past Projects

2019-2022. GRAMO. Identification of biomarkers of early damage in adipose tissue in relation to human chronic exposure to environmental pollutants. AAP Junta Andalucia 2019 - LABERCA-University of Granada, ESP 110 k eur [Partner].

2019-2022. EndoxOMICs-β: Mise en place d'une nouvelle approche analytique intégrée pour l'identification simultanée de signatures d'exposition et biomarqueurs d'effet – application à la compréhension du lien exposome - endométriose et infertilité associée. (Etoiles Montantes 2019, Région Pays de la Loire, 110k euros).

2018-2020. POLOB. Lipophilic organic pollutants and morbid obesity: Kinetics of release and influence on the metabolic syndrome before and after bariatric surgery. 12-API-01/N° ID-RCB : 2013-A00396-39. CENTRE HOSPITALIER UNIVERSITAIRE DE NICE – LABERCA.

2017-2021. ED-Cancer. Exposure to Endocrine Disrupting Chemicals and Risk of Hormone-Related Cancers. INSRM Environnement et Cancer. Plan Cancer 2014-2019. INSERM-LABERCA

2019-2021. NutPOP. Exploring the pollutants-micronutrients interface during early pregnancy and their impact on child growth and metabolic status. IS Global-Laberca. (Co-Principal Investigator).

2018-2021. ENDOMET2. Étude du lien entre l'exposition aux perturbateurs endocriniens (EDC) et l'endométriose : une approche intégrative. Pays de la Loire CPER 2014-2020. LABERCA-CHU Nantes.

2014-2019. METAPOP, Impact des Polluants Organiques Persistants sur le potentiel métastatique des tumeurs mammaires malignes n° 11-MRES-PNRPE-5-CVS-031 Chorus n° 2100532530. Collab. INSERM-LABERCA.

2015-2016. Obesogenic and diabetogenic risk assessment of chemical contaminants. Office of Environmental Health Hazard Assessment award 13-E0014-1, and National Institutes of Health (ES023513). Davis, California.

2013-2014. "ECsafeSEAFOOD. Contaminants in seafood and their impact on public health (The Ocean of Tomorrow)" Collaborative project. THEME [KBBE.2012.2.4-01]. Nº 311820. 7th Framework Programme . European Commission. University "Rovira i Virgili"

List of doctoral grants

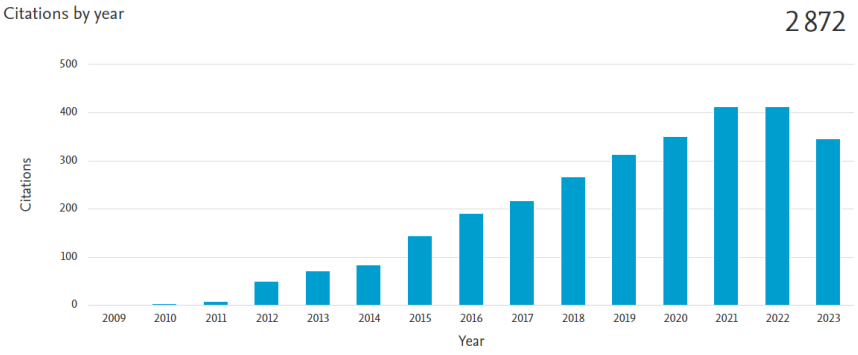
- Bourse de Thèse Doctorale co-financé Région Pays de la Loire-INRAE (Etienne BABIN, 2021-2024) 130k eur [co-PI]

- Bourse de Thèse Doctorale Région Pays de la Loire-ONIRIS (Komodo MATTA, 2019-2022) 130k eur [co-PI]

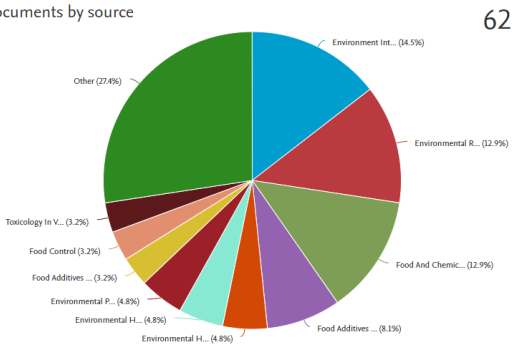
4.3 List of publications in scientific journals

Total publications 62, Scopus h-index=27

Scopus profil link: https://www.scopus.com/authid/detail.uri?authorId=36021084400



Source	Documents
Environment International	9
Environmental Research	8
Food And Chemical Toxicology	8
Food Additives And Contaminants Part A	5
Environmental Health A Global Access Science Source	3
Environmental Health Perspectives	3
Environmental Pollution	3
Food Additives And Contaminants Part B Surveillance	2



62. Babin, É, **Cano-Sancho, G**, Vigneau, E, Antignac, JP. 2023. A review of statistical strategies to integrate biomarkers of chemical exposure with biomarkers of effect applied in omic-scale environmental epidemiology. Environmental Pollution, 330:121741. doi: 10.1016/j.envpol.2023.121741, IF:8.9.

61. **Cano-Sancho, G**, Warembourg, C, Güil, N, Stratakis, N, Lertxundi, A, Irizar, A, Llop, S, Lopez-Espinosa, MJ, Basagaña, X, González, JR, Coumoul, X, Fernández-Barrés, S, Antignac, JP, Vrijheid, M, Casas, M. 2023. Nutritional Modulation of Associations between Prenatal Exposure to Persistent Organic Pollutants and Childhood Obesity: A Prospective Cohort Study. Environmental Health Perspectives, 131(3):37011. doi: 10.1289/EHP11258, IF: 11.035. **[Selected Publication 1, copy in Annex 2]**

60. Gallardo, JA, Marín, S, Ramos, AJ**, Cano-Sancho**, G, Sanchis, V. 2022. Occurrence and Dietary Exposure Assessment to Enniatin B through Consumption of Cereal-Based Products in Spain and the Catalonia Region. Toxins, 29;15(1):24. doi: 10.3390/toxins15010024, IF: 5.075.

59. Moriceau, MA, **Cano-Sancho, G**, Kim, M, Coumoul, X, Emond, C, Arrebola, JP, Antignac, JP, Audouze, K, Rousselle, C. 2022. Partitioning of Persistent Organic Pollutants between Adipose Tissue and Serum in Human Studies. Toxics, 31;11(1):41. doi: 10.3390/toxics11010041, IF: 4.471.

58. Benoit, L, Koual, M, Tomkiewicz, C, Bats, AS, Antignac, JP, Coumoul, X, Barouki, R, **Cano-Sancho, G**. 2022. Impact of mixtures of persistent organic pollutants on breast cancer

aggressiveness. Environmental International, 170:107615. doi: 10.1016/j.envint.2022.107615, IF: 13.352.

57. Bajard L, Adamovsky O, Audouze K, Baken K, Barouki R, Beltman JB, Beronius A, Bonefeld-Jørgensen EC, **Cano-Sancho G**, de Baat ML, Di Tillio F, Fernández MF, FitzGerald RE, Gundacker C, Hernández AF, Hilscherova K, Karakitsios S, Kuchovska E, Long M, Luijten M, Majid S, Marx-Stoelting P, Mustieles V, Negi CK, Sarigiannis D, Scholz S, Sovadinova I, Stierum R, Tanabe S, Tollefsen KE, van den Brand AD, Vogs C, Wielsøe M, Wittwehr C, Blaha L. 2023. Application of AOPs to assist regulatory assessment of chemical risks - Case studies, needs and recommendations. Environmental Research, 15;217:114650. doi: 10.1016/j.envres.2022.114650, IF: 8.431.

56. Lefebvre, T, Fréour, T, Ploteau, S, Marchand, P, Le Bizec, B, Antignac, JP**, Cano-Sancho, G**. 2023. Mixtures of persistent organic pollutants and ovarian function in women undergoing IVF. Reproductive Biomedicine Online, 2023 46(1):129-137. doi: 10.1016/j.rbmo.2022.09.015, IF: 4.567.

55. Güil-Oumrait, N, **Cano-Sancho, G**, Montazeri, P, Stratakis, N, Warembourg, C, Lopez-Espinosa, MJ, Vioque, J, Santa-Marina, L, Jimeno-Romero, A, Ventura, R, Monfort, N, Vrijheid, M, Casas, M. 2022. Prenatal exposure to mixtures of phthalates and phenols and body mass index and blood pressure in Spanish preadolescents. Environmental International, 15;169:107527. doi: 10.1016/j.envint.022.107527, IF: 13.352.

54. Lefebvre, T, Fréour, T, Duval, G, Ploteau, S, Marchand, P, Le Bizec, B, Antignac, JP, Cano-Sancho, G. 2022. Associations between internal concentrations of fluorinated and organochlorinated chemicals in women and in vitro fertilization outcomes: A multi-pollutant study. Environmental Pollution, 7; 313:120087. doi: 10.1016/j.envpol.2022.120087, IF: 9.988. **[Selected Publication 2, copy in Annex 2]**

53. Frenoy, P, Marques, C, Fiolet, T, Cano-Sancho, G, Severi, G, Mancini, FR. Positive association between dietary exposure to polybrominated diphenyl ethers and breast cancer risk in the French E3N cohort: The role of vegetable oil consumption. Environment International, 167:107444, doi: 10.1016/j.envint.2022.107444, IF: 12.25.

52. Gallardo, JA, Marin, S, Ramos, AJ, **Cano-Sancho, G**, Sanchis, V. 2022. Deterministic and Probabilistic Dietary Exposure Assessment to Deoxynivalenol in Spain and the Catalonia Region. Toxins, 20;14(7):506. doi: 10.3390/toxins14070506, IF: 5.075.

51. Frenoy, P, Perduca, V, **Cano-Sancho, G**, Antignac, JP, Severi, G, Mancini, FR. Application of two statistical approaches (Bayesian Kernel Machine Regression and Principal Component Regression) to assess breast cancer risk in association to exposure to mixtures of brominated flame retardants and per- and polyfluorinated alkylated substances in the E3N cohort. Environmental Health, 26;21(1):27 doi: 10.1186/s12940-022-00840-4, IF: 5.984.

50. Omichessan, H, Perduca, V, Polidoro, S, Kvaskoff, M, Truong, T, **Cano-Sancho, G**, Antignac, JP, Baglietto, L, Mancini, FR, Severi, G. 2022. Associations between plasma levels of brominated flame retardants and methylation of DNA from peripheral blood: A cross-sectional study in a cohort of French women. Environmental Research, 3;210:112788 doi: 10.1016/j.envres.2022.112788, IF:6.498.

49. Matta, K, Lefebvre, T, Vigneau, E, Cariou, V, Marchand, P, Guitton, Y, Royer, AL, Ploteau, S, Le Bizec, B, Antignac, JP, **Cano-Sancho, G**. 2021.Associations between persistent organic pollutants and endometriosis: A multiblock approach integrating metabolic and cytokine profiling. Environmental International, 158:106926. doi: 10.1016/j.envint.2021.106926, IF: 12.25. **[Selected Publication 3, copy in Annex 2]**

48. Perrot-Applanat, M, Pimpie, C, **Cano-Sancho, G**, Antignac, JP, Pocard, M. 2021. Detection of Persistent Organic Pollutants in Omental Adipose Tissue from Patients with Diffuse-Gastric Cancer: A Pilot Study. Cancers, 29;13(19):4874, doi: 10.3390/cancers13194874, IF: 6.64.

47. Lefebvre, T, Fréour, T, Ploteau, S, Le Bizec, B, Antignac, J-P, **Cano-Sancho, G**. 2021. Associations between human internal chemical exposure to Persistent Organic Pollutants (POPs) and In Vitro Fertilization (IVF) outcomes: Systematic review and evidence map of human epidemiological evidence Reproductive Toxicology, doi: 10.1016/j.reprotox.2021.09.005, IF: 3.143.

46. Matta, K, Koual, M, Ploteau, S, Coumoul, X, Audouze, K, Bizec, BL, Antignac, J-P, **Cano-Sancho, G**. 2021. Associations between exposure to organochlorine chemicals and endometriosis: A systematic review of experimental studies and integration of epidemiological evidence. Environmental Health Perspectives, doi: 10.1289/EHP8421, IF: 11.035. **[Selected Publication 4, copy in Annex 2]**

45. Fénichel, P, Coquillard, P, Brucker-Davis, F, Marchand, P, **Cano-Sancho, G**, Boda, M, Antignac, J-P, Iannelli, A, Gugenheim, J, Le Bizec, B, Chevalier, N. 2021. Sustained bloodstream release of persistent organic pollutants induced by extensive weight loss after bariatric surgery: Implications for women of childbearing age. Environment International, doi: 10.1016/j.envint.2021.106400, IF : 7.6.

44. Marín, S., Ramos, A.J., Sanchis, V., **Cano-Sancho, G**. 2021. An overview of mycotoxin biomarker application in exposome-health studies. Current Opinion in Food Science, doi: 10.1016/j.cofs.2020.12.011, IF: 9.8.

43. **Cano-Sancho, G**; Casas, M. 2021. Interactions between environmental pollutants and dietary nutrients: current evidence and implications in epidemiological research. Journal Of Epidemiology And Community Health, doi:10.1136/jech-2020-213789, IF: 3.34

42. Koual, M; Tomkiewicz, C; **Cano-Sancho, G**; Antignac, JP; Bats, AS; Coumoul, X. 2020. Environmental chemicals, breast cancer progression and drug resistance. Environmental Health, doi:10.1186/s12940-020-00670-2, IF: 4.7.

41. Elmore, SE; **Cano-Sancho, G**; La Merrill, MA. 2020. Disruption of normal adipocyte development and function by methyl- and propyl- paraben exposure. Toxicology Letters, doi:10.1016/j.toxlet.2020.09.009, IF: 4.7.

40. Mancini, FR; **Cano-Sancho, G**; Mohamed, O; Cervenka, I; Omichessan, H; Marchand, P; Boutron-Ruault, MC; Arveux, P; Severi, G; Antignac, JP; Kvaskoff, M. 2020. Plasma concentration of brominated flame retardants and postmenopausal breast cancer risk: a nested case-control study in the French E3N cohort. Environmental Health, doi:10.1186/s12940-020-00607-9, IF: 4.7.

39. Matta, K; Vigneau, E; Cariou, V; Mouret, D; Ploteau, S; Le Bizec, B; Antignac, JP; **Cano-Sancho, G**. 2020. Associations between persistent organic pollutants and endometriosis: A multipollutant assessment using machine learning algorithms. Environmental Pollution, doi:10.1016/j.envpol.2020.114066, IF : 6.8.

38. **Cano-Sancho, G**; Alexandre-Gouabau, MC; Moyon, T; Royer, AL; Guitton, Y; Billard, H; Darmaun, D; Roze, JC; Boquien, CY; Le Bizec, B; Antignac, JP. 2020. Simultaneous exploration of nutrients and pollutants in human milk and their impact on preterm infant growth: An integrative cross-platform approach. Environmental Research, doi:10.1016/j.envres.2019.109018, IF: 5.7. **[Selected Publication 5, copy in Annex 2]**

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11. **Cano-Sancho, G**; Ramos, AJ; Marin, S; Sanchis, V. 2012. Occurrence of fumonisins in Catalonia (Spain) and an exposure assessment of specific population groups. Food Additives And Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment, doi:10.1080/19440049.2011.644813 IF: 2.22.

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4. **Cano-Sancho, G**; Marin, S; Ramos, AJ; Peris-Vicente, J; Sanchis, V. 2010. Occurrence of aflatoxin M-1 and exposure assessment in Catalonia (Spain). Revista Iberoamericana De Micologia, doi:10.1016/j.riam.2010.05.003 IF: 1.7.

3. **Cano-Sancho, G**; Marin, S; Ramos, AJ; Sanchis, V. 2010. Biomonitoring of Fusarium spp. Mycotoxins: Perspectives for an Individual Exposure Assessment Tool. Food Science And Technology International, doi:10.1177/1082013210368884 IF:0.784

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1. **Cano-Sancho, G**; Marin, S; Ramos, AJ; Sanchis, V. 2009. Survey of patulin occurrence in apple juice and apple products in Catalonia, Spain, and an estimate of dietary intake. Food Additives & Contaminants Part B-Surveillance, doi:10.1080/026520309028977 IF:2.6.

4.4 Editorial activities

Reviewer for the following Scientific Journals, the « n » indicates the number of articles reviewed between 2017 and 2023 : Environment International (n=15) Open Access Journal, Environmental Pollution (n=2), Environmental Health (n=3), Frontiers Endocrinology (n=1), Food and Chemical Toxicology (n=2), Human Reproduction Update (n=1).

4.5 Book publications

2. Cano-Sancho, G., Marin, S., Ramos, A.J., Sanchis, V. 2009. Micotoxines. Estudi de dieta total a Catalunya, 2008-2009. Agència Catalana de Seguretat Alimentaria (ACSA), Generalitat de Catalunya, Barcelona, Spain. [eBOOK: B. 27368-2012]. https://acsa.gencat.cat/web/.content/_Publicacions/Estudis_de_dieta_total/micotoxines/estudi_micotoxines_castella.pdf

1. Marin, S, Cano-Sancho, G. 2020. Chapter 17. Examples of quantitative mycotoxin risk assessments: Use and application in risk management. In: Risk Assessment Methods for Biological and Chemical Hazards in Food. 2020 CRC Press, Boca Raton. https://doi.org/10.1201/9780429083525

4.6 Communication in scientific congresses

Conference and Workshop Presentations (Total = 37; Invited = 6).

37. **German Cano-Sancho**, Thomas Moyon, Hélène Billard, Cécile Boscher, Laure Simon, Jean-Christophe Roze, Blandine De Lauzon-Guillain, Marie-Cécile Alexandre-Gouabau, Jean-Philippe Antignac, Evelyne Vigneau, Clair-Yves Boquien. Dietary patterns of mothers of preterm infants and its relationship with levels of persistent organic pollutants in their milk during the first month postpartum. 7th International EMBA Congress 25-27 October Madrid, Spain [POSTER]

36. **German Cano-Sancho**, Jean-Philippe Antignac, Le niveau de preuve des études en santé environnementale : une approche développée par le National Toxicology Program (NTP) du Office of Health Assessment and Translation (OHAT) 61ème congrès annuel de la STC Lyon, 11 octobre au 13 octobre 2023 Lyon [INVITED ORAL PRESENTATION]

35. J.A. Gallardo, **G. Cano-Sancho**, A.J. Ramos, S. Marín, V. Sanchis. Exposure assessment of Catalonian and Spanish population to Deoxynivalenol and Enniatin B through cereal-based food. MICOFOOD 28-30th June Valencia, Spain 2023

34. Anne-Lise Royer; Tiphaine Lefebvre; Thomas Fréour ; Stéphane Ploteau ; Philippe Marchand ; Bruno Le Bizec; Yann Guitton; Jean-Philippe Antignac; **German Cano Sancho**. Linking blood oxylipin profiles to internal POPs exposure levels in women undergoing in vitro fertilization. 15émes journées scientifiques du RFMF qui se tiendront du 24 au 26 Mai 2023 à Perpignan [POSTER]

33. **German Cano-Sancho**. Perturbateurs endocriniens et endométriose L'endométriose, une maladie liée à l'environnement et un combat politique. Journées de Suivi Nesting / Ma Maison Ma Santé 9 de mars 2023 WECF France [INVITED ORAL PRESENTATION]

32. **German Cano-Sancho**, Charline Warembourg, Nuria Güil, Aitana Lertxundi, Amaia Irizar, Sabrina Llop, Mª Jose Lopez ,Xavier Basagaña, Juan Ramon González, Sílvia Fernandez, Jean-Philippe Antignac, Martine Vrijheid, Maribel Casas. Interactions between persistent organic pollutants and nutritional biomarkers on birth weight 18th Jornadas Cientificas INMA – 2023, February 7-9 Barcelona [ORAL PRESENTATION]

31. Manon Campas, Tiphaine Lefebvre, Yan Guitton, Stéphane Ploteau, Bruno Le Bizec, Jean-Philippe Antignac, **German Cano Sancho**, Pascal de Tullio. Application of NMR-based metabolomics to the study of endometriosis and infertility in French women 18th Annual Conference of the Metabolomics Society – Metabolomics 2022, June 19-23 Valencia [POSTER PRESENTATION]

30. **German Cano Sancho**; Tiphaine Lefebvre; Manon Campas; Komodo Matta; Yann Guitton; Gauthier Duval ; Stéphane Ploteau ; Philippe Marchand ; Bruno Le Bizec; Pascal de Tullio; Jean-Philippe Antignac; Metabolic profiles of French women with and without endometriosis and infertility: a metabolomics-exposomic multi-platform analysis to assess the pollutant modulation. Annual Conference of the Metabolomics Society – Metabolomics 2022, June 19-23 Valencia [POSTER PRESENTATION]

29. **German Cano-Sancho**, Charline Warembourg, Nuria Güil, Aitana Lertxundi, Amaia Irizar, Sabrina Llop, Mª Jose Lopez ,Xavier Basagaña, Juan Ramon González, Sílvia Fernandez, Jean-Philippe Antignac, Martine Vrijheid, Maribel CasasNutritional modulation of associations between persistent organic pollutants during pregnancy and childhood obesity. European Human Exposome Nertwork Scientific Meeting 24-25 May 2022 Barcelona [ORAL PRESENTATION]

28. **German Cano-Sancho.** Évaluation de l'impact des contaminants alimentaires durant la période périnatale. CONGRÈS ANNUEL MIBIOGATE 10-11 Mars 2022 Nantes [Invited Speaker]

27. **German Cano-Sancho**, Charline Warembourg, Nuria Güil, Aitana Lertxundi, Amaia Irizar, Sabrina Llop, Mª Jose Lopez, Xavier Basagaña, Juan Ramon González, Sílvia Fernandez, Jean-Philippe Antignac, Martine Vrijheid, Maribel Casas Interactions between micronutrients and persistent organic pollutants during pregnancy: impacts on childhood obesity, 2022 Prenatal Programming and Toxicity (PPTOX) meeting PPTOX-VII, 10-13th January 2022 [POSTER PRESENTATION]

26. Nuria Güil-Oumrait, **German Cano-Sancho**, Nikos Stratakis, Charline Warembourg, María José Lopez, Jesús Vioque López, Loreto Santa-María, Alba Jimeno, Martine Vrijheid, Maribel Casas Prenatal Exposure to Mixtures of Non-persistent Chemicals and Body Mass Index and Blood Pressure in Spanish Preadolescents, 2022 Prenatal Programming and Toxicity (PPTOX) meeting PPTOX-VII, 10-13th January 2022 [POSTER PRESENTATION]

25. **German Cano-Sancho**, Charline Warembourg, Nuria Güil, Aitana Lertxundi, Amaia Irizar, Sabrina Llop, Mª Jose Lopez ,Xavier Basagaña, Juan Ramon González, Sílvia Fernandez, Jean-Philippe Antignac, Martine Vrijheid, Maribel Casas Nutritional modulation of associations between persistent organic pollutants during pregnancy and childhood obesity 17ª Jornadas Científicas INMA 2021, San Sebastian, Spain, [ORAL PRESENTATION, not presenter]

24. **Cano-Sancho, German** ; Marchand, Philippe ; Le Bizec, Bruno ; Antignac, Jean-Philippe. 2021. Identification of chemicals in the "omic" era. Challenges and opportunities. 10th International Breast Cancer Symposium Nantes/Online [Invited KEYNOTE speaker]

23. **German Cano-Sancho**. A novel landscape in chemical risk assessment called Exposome: current applications and opportunities. International Conference on Food Contaminants 2021, 27 / 28 September 2021. Portugal, online. [KEYNOTE speaker]

22. **German Cano-Sancho,** Charline Warembourg, Nuria Güil, Aitana Lertxundi, Amaia Irizar, Sabrina Llop, Mª Jose Lopez ,Xavier Basagaña, Juan Ramon González, Sílvia Fernandez, Jean-Philippe Antignac, Martine Vrijheid, Maribel Casas, Impact of maternal nutritional status during pregnancy on the associations between persistent organic pollutants and childhood overweight. 33rd Annual Conference of the International Society for Environmental Epidemiology / ISEE 2021 will take place on August 23-26, 2021, New York-Online [POSTER/ORAL PRESENTATION]

21. Matta Komodo, Lefebvre Tiphaine, Guitton Yann, Marchand Philippe, Le Bizec Bruno, Ploteau Stéphane, Antignac Jean-Philippe, **Cano-Sancho German**. Associations between persistent organic pollutants exposure and endometriosis: integrating the exposome and metabolome. Journées Scientifiques de l'Ecole Doctorale Biologie Santé à L'Université Bretagne Loire, Virtual Meeting, 10 December 2020. [POSTER PRESENTATION]

20. Matta Komodo, Ploteau Stéphane, Guitton Yann, Ouzia Sadia, Le Bizec Bruno, Antignac Jean-Philippe, **Cano-Sancho German**. Quantitative metabolomics analysis reveals altered lipid profile in women with endometriosis: a pilot case-control study. European RFMF Metabomeeting, Toulouse, France. Jan 2020 [ORAL PRESENTATION, not presenter]

19. Matta Komodo, Lefebvre Tiphaine, Guitton Yann, Marchand Philippe, Le Bizec Bruno,Ploteau Stéphane, Antignac Jean-Philippe, **Cano-Sancho German**. Linking biomarkers of environmental chemical exposure and endometriosis: integrating the exposome and metabolome in the EndoxOmics-β Study. 32nd Annual Conference of the International Society for Environmental Epidemiology (ISEE 2020), Virtual meeting, 24-27 August 2020. [ORAL PRESENTATION, not presenter]

18. **Cano-Sancho G** ; Marchand Philippe ; Le Bizec, Bruno ; Antignac, Jean-Philippe. 2019. Identification des facteurs de risque chimiques environnementaux de cancer à l'ère OMIQUE: défis et opportunités. 13èmes Journées du Cancéropôle Grand Ouest. 1-2 July TOURS 2019.

17. Mancini FR, **Cano-Sancho G**, Gambaretti J, Marchand P, Antignac JP, Kvaskoff M. 2018. Circulating levels of perfluorinated compounds and breast cancer risk: evidence from a nested case-control study. European Congress of Epidemiology, 4-6 July Lyon 2018

16. **Cano-Sancho**, G, Ploteau, S, Volteau, C, Legrand, A, Vénisseau, A, Vaccher, V, Marchand, P, Le Bizec, B, Antignac, JP. 2017. Influence of the circulating lipids component and options for assessing the link between internal exposure to POPs and health: a compared approach applied to endometriosis. 37th International Symposium on Halogenated Persistent Organic Pollutants (POPs) - DIOXIN 2017.

15. **Cano-Sancho, G**, Ploteau, S, Volteau, C, Legrand, A, Vénisseau, A, Vaccher, V, Marchand, P, Le Bizec, B, Antignac, JP. 2017. Assessing the associations between persistent organic pollutants and endometriosis: single versus multi-pollutant approaches applied to a first case-control study. 9th Copenhagen Workshop on Endocrine Disrupters COW2017.

14. **Cano-Sancho, G**., Perello G, Nadal M, Domingo JL. 2014. Co-occurrence of the mycotoxin deoxynivalenol and trace metals in vegetarian culinary specialities. Food Analysis Congress.

13. Jacobs S, Sioen I, Pieniak Z, De Henauw S, Maulvault AL, Reuver M, Fait G, **Cano-Sancho, G**., Verbeke W.European consumers' benefit-risk perception and the association with their consumption of seafood. WEFTA, 44th Annual meeting.

12. **Cano-Sancho, G.**, Ramos, A.J., Sanchis, V., Marín, S., Fernández-Cruz, M.L. 2012. Cytotoxic effects of the simultaneous exposure of Caco-2 cells to mycotoxins and antioxidants. World Mycotoxin Forum meets IUPAC.

11. **Cano-Sancho, G**., Sanchis, V., Ramos, A.J., Marin, S. 2012. Mycotoxin in special commodities: Gluten-free and ethnic foods. International MPU Workshop 2012.

10. Vidal, A., Marin, S., Ramos, A.J., **Cano-Sancho, G**., Sanchis, V. 2012. Mycotoxin contamination in wheat and oat based bran supplements from the Spanish retail market. International MPU Workshop 2012.

9. **Cano-Sancho, G**., Marin, S., Ramos, A.J., Sanchis, V. 2012. Exposure assessment of Catalonian (Spain) population to Fusarium mycotoxins. 2 International CIGR Workshop on Food Safety: Technologies and Innovations Applied to Food Safety.

8. **Cano-Sancho, G**., Estiarte, N., Vidal, A., Marín, S. 2012. T-2 and HT-2 in Catalonia (Spain): occurrence and exposure assessment. 2nd International CIGR Workshop on Food Safety: Technologies and innovations applied to food safety.

7. **Cano-Sancho, G**., Ramos, A.J., Sanchis, V., Marin, S. 2011. Dietary intake of zearalenone in Catalonia (Spain). MYCORED ARGENTINA - ISM 2011 Conference - Strategies to reduce the impact of mycotoxins in Latin America in a global context.

6. Cela, E.Mª.G., **Cano-Sancho, G**., Marín, S., Ramos, A.J., Sanchís, V. 2010. Incidencia de Aflatoxina M1 en productos lácteos. Medida de la exposición en Cataluña. XVII Congreso Nacional de Microbiología de los Alimentos.

5. Sanchis, V., **Cano-Sancho, G**., Ramos, A.J., Marín, S. 2010. Ocurrence of mycotoxins in Catalonia (Spain) and global exposure assessment. Workshop Mycotoxicological risks in Mediterranean countries.

4. **Cano-Sancho, G**., Sanchis, V., Ramos, A.J., Marín, S. 2010. Dietary intake of the mycotoxin deoxynivalenol by the catalonia (Spain) population. Workshop Mycotoxicological risks in Mediterranean countries.

3. **Cano-Sancho, G**., Ramos, A.J., Marín, S., Sanchis, V. 2010. Dietary intake of aflatoxins by Catalonian (Spain) population. VI Latin American Congress of Mycotoxicology.

2. Sanchis, V., **Cano-Sancho, G**., Ramos, A.J., Marin, S. 2009. Occurrence of fumonisins in corn based food and exposure assessment of population from Catalonia (Spain). ISM Conference 2009. Worldwide Mycotoxin Reduction in Food and Feed chains.

1. **Cano-Sancho, G**., Sanchis, V., Ramos, A.J., Marín, S. 2009. Ocurrence of patulin in apple juice and apple products and exposure assessment of the population from Catalonia (Spain). The SAFE Consortium International Congress on Food Safety.

4.7 Scientific Collaborations

Regional and National collaborations

- Stéphane Ploteau, Thomas Freour, CHU Nantes Service AMP et Service Gynécologie-Obstétrique, Nantes
- Marina Kvaskoff, Francesca Mancini. Exposome and Epidemiology of Endometriosis, INSERM, France (epidemiology, exposome)
- Mathilde Touvier, CRESS, INSERM, INRAE, France (nutritional epidemiology)
- Xavier Coumoul, Karine Audouze, Toxicology, Environmental Toxicity, Therapeutic Targets, cellular Signaling and Biomarkers Team, INSERM, France (toxicology, systems toxicology)
- Nathalie Poupin, Fabien Jourdan, ToxAlim, Toulouse, France (bioinformatics, systems metabolomics)

International collaborations

- Martine Vrijheid, Exposome Research and Environmental Epidemiology, ISGlobal, Spain (epidemiology, exposome)
- Pascal de Tullio, University of Liege, Belgium (reproductive metabolomics)
- Michelle La Merrill, Toxicology of Endocrine Disruptors and Public Health, Environmental Toxicology Department, University of California at Davis, US (toxicology, systems toxicology)
- Maykel Hernandez-Mesa, University of Granada, Spain (chemical analysis, exposomic methods)
- Juan Pedro Arrebola, Instituto Carlos III, Spain (environmental epidemiology)

ANNEX 1. LIST OF REFERENCES

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ANNEX 2. SELECTED PUBLICATIONS

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Nutritional Modulation of Associations between Prenatal Exposure to Persistent Organic Pollutants and Childhood Obesity: A Prospective Cohort Study

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Background: Prenatal exposure to persistent organic pollutants (POPs) may contribute to the development of childhood obesity and metabolic disorders. However, little is known about whether the maternal nutritional status during pregnancy can modulate these associations.

Objectives: The main objective was to characterize the joint associations and interactions between prenatal levels of POPs and nutrients on childhood obesity.

Methods: We used data from to the Spanish INfancia y Medio Ambiente–Environment and Childhood (INMA) birth cohort, on POPs and nutritional biomarkers measured in maternal blood collected at the first trimester of pregnancy and child anthropometric measurements at 7 years of age. Six organochlorine compounds (OCs) [dichlorodiphenyldichloroethylene, hexachlorobenzene (HCB), β-hexachlorocyclohexane (β-HCH) and polychlorinated biphenyls 138, 153, 180] and four per- and polyfluoroalkyl substances (PFAS) were measured. Nutrients included vitamins (D, B12, and folate), polyunsaturated fatty acids (PUFAs), and dietary carotenoids. Two POPs–nutrients mixtures data sets were established: *a*) OCs, PFAS, vitamins, and carotenoids ($n = 660$), and *b*) OCs, PUFAs, and vitamins ($n = 558$). Joint associations of mixtures on obesity were characterized using Bayesian kernel machine regression (BKMR). Relative importance of biomarkers and two-way interactions were identified using gradient boosting machine, hierarchical group lasso regularization, and BKMR. Interactions were further characterized using multivariate regression models in the multiplicative and additive scale.

Results: Forty percent of children had overweight or obesity. We observed a positive overall joint association of both POPs–nutrients mixtures on overweight/obesity risk, with HCB and vitamin B12 the biomarkers contributing the most. Recurrent interactions were found between HCB and vitamin B12 across screening models. Relative risk for a natural log increase of HCB was 1.31 (95% CI: 1.11, 1.54, $p_{\text{Interaction}} = 0.02$) in the tertile 2 of vitamin B12 and in the additive scale a relative excess risk due to interaction of 0.11 (95% CI: 0.02, 0.20) was found. Interaction between perfluorooctane sulfonate and β-cryptoxanthin suggested a protective effect of the antioxidant on overweight/obesity risk.

Conclusion: These results support that maternal nutritional status may modulate the effect of prenatal exposure to POPs on childhood overweight/obesity. These findings may help to develop a biological hypothesis for future toxicological studies and to better interpret inconsistent findings in epidemiological studies. https://doi.org/10.1289/EHP11258

Introduction

Growing evidence supports that environment insults and nutrition during the early stages of development may influence subsequent health during childhood, including obesity and metabolic diseases.[1,2] Childhood obesity remains a public health priority owing to its high prevalence, associated risk of comorbidities, and high societal costs,[3,4] and has been linked to a growing list of environmental factors including synthetic chemicals.[5,6] Persistent organic pollutants (POPs) represent a vast family of chemicals characterized by their hydrophobicity, stability, and capacity to bioaccumulate across the trophic chains and are widespread in the fatty tissues of populations across the globe.[7–9] Some POPs, such as the pesticide dichlorodiphenyltrichloroethane (p,p'-DDT), its main metabolite dichlorodiphenyldichloroethane (p,p'-DDE), or hexachlorobenzene (HCB), have been associated with obesity or metabolic disruption in human prospective studies and this has been supported by several experimental studies.[10–14]

Diet is a major pathway of exposure to many POPs,[15] but it can also modulate the effect of toxicants by means of bioactive nutrients,[16] thus becoming a source of heterogeneity or confounding bias in environmental epidemiology if not properly addressed.[17] Bioactive lipids and lipophilic nutrients are specially relevant for POPs because their shared mechanisms of uptake, transport, and metabolism, and targeting similar molecular pathways, resulting in a large potential to confound, jointly affect, or interact in multiple health outcomes.[18] This may be the case of carotenoids, lipophilic vitamins or ω3 poly unsaturated fatty acids (PUFAs) when assessing the obesogenicity of POPs, owing to their reported *in vitro* bioactivity on lipogenic and lipolytic pathways.[19–21] The interactive effect of POPs with

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ω3-PUFAs has been observed in experimental studies, counterbalancing their effects on insulin resistance prevention.[19–21]

Previous studies within the Spanish longitudinal INfancia y Medio Ambiente–Environment and Childhood (INMA) birth cohort have reported positive associations between prenatal exposure to organochlorine compounds (OCs) with higher offspring obesity risk,[5,14,22,23] but mild or null associations for per- and polyfluoroalkyl substances (PFAS).[24] Although some studies have assessed the joint associations of prenatal mixtures of toxic metals and nutritional factors on child outcomes,[25] to the best of our knowledge, no studies have attempted to assess the joint associations of POPs and nutrients on overweight/obesity to date. Thus, built on the hypothesis that the health effects of POPs may be modulated by bioactive lipids and lipophilic nutrients,[18] this study extends the previous INMA work to characterize the joint effect of prenatal POPs and lipophilic micronutrients and/or PUFAs on childhood obesity risk. Considering the high-dimensional nature of interaction evaluations, the present study develops a comprehensive exploratory framework within a hypothesis-driven context, intended to answer major questions in epidemiological mixture analyses.[26–29] More specifically, those questions included *a*) "What is the association of prenatal POPs and nutrients as a mixture on childhood overweight/obesity risk?", and *b*) "Are there interactions between biomarkers of nutritional status in the associations between POPs and overweight/obesity risk?"

Methods

Study Population

Data from the Spanish INMA birth cohort study were used for the present analysis, extensively described elsewhere.[30] Briefly, the INMA study is a network of population-based birth cohorts conducted in seven different regions from Spain, four of them (Asturias, Gipuzkoa, Sabadell, and Valencia) sharing the recruitment timeframe and protocol to ensure harmonization. For the present study, we considered three of those regions (Gipuzkoa, Sabadell, and Valencia) owing to the data availability, representing a total of 2,150 pregnant women recruited at the first trimester of pregnancy (weeks 10–13 of gestation) from 2003 to 2008. To be eligible, women must be at least 16 y old and present singleton pregnancy, no communication barrier, need no reproductive assistance, and be giving birth in the reference hospital.[30] Follow-up visits were conducted when children were 1–1.5, 4, 7, and 11 y old, yet the study is still ongoing. When the children were 7 years of age, 1,394 mother–child pairs participated in the follow-up. The study was approved by the ethical committees of the centers involved in the study (listed in the "Acknowledgments" section). Written informed consent was obtained from the parents of all children. In the present analysis, we included mother–child pairs with information on blood biomarkers of POPs (OCs and PFAS) exposure and nutrient intake (vitamins, PUFAs, and carotenoids) during pregnancy and child anthropometric measurements at 7 years of age (Figure S1). A total of 1,241 mothers had information on OCs and vitamins and child obesity outcomes (Figure S1). However, because not all women had available information of all POPs and nutrients, we generated two consolidated data sets for the mixture analysis: the ANTIOX data set with OCs, PFAS, vitamins, and carotenoids ($n = 660$ with 30 variables) and the PUFA data set consisting of OCs, vitamins, and PUFAs ($n = 558$ with 14 variables), see details in Table 1. Among both data sets there was an overlap of 241 mother–child pairs. The list of compounds included within each data set is detailed in Table 1.

Table 1. Distribution [median (25th–75th percentile)] of persistent organic pollutants and nutrients for each data set measured in blood from mothers from INMA Guipuzkoa, Sabadell, and Valencia cohorts (Spain).

Maternal biomarkers (units)	ANTIOX $N = 660$	PUFA $N = 558$
Organochlorine compounds (ng/g lipid)		
HCB	55.2 (31.3–91.0)	39.0 (29.2–78.7)
β-HCH	23.6 (6.71–35.1)	30.5 (15.3–38.4)
p,*p*′-DDE	134.0 (85.5–234)	122.0 (79.7–202)
PCB138	33.5 (23.5–45.6)	25.5 (16.0–37.6)
PCB153	52.5 (37.3–71.6)	42.9 (29.0–61.8)
PCB180	39.2 (26.7–55.6)	30.2 (20.2–43.1)
Per- and polyfluoroalkyl substances (ng/mL)		
PFOA	2.10 (1.47–2.91)	—
PFOS	5.88 (4.49–7.66)	—
PFHxS	0.483 (0.37–0.66)	—
PFNA	0.582 (0.43–0.76)	—
Vitamins		
B12 (pmol/L)	397 (310–519)	341 (271–428)
D (mmol/L)	30.3 (22.9–37.6)	29.7 (21.2–37.5)
Folate (mmol/L)	17.9 (12.7–25.7)	14.2 (9.70–23.1)
Polyunsaturated fatty acids (% of fatty acids)		
LA	—	30.9 (25.2–34.2)
ALA	—	0.3 (0.2–0.3)
EPA	—	0.3 (0.2–0.5)
DHA	—	2.9 (2.3–4.1)
AA	—	8.0 (6.8–9.6)
Carotenoids (μmol/L)		
γ-Tocopherol	1.49 (1.24–1.87)	—
α-Tocopherol	30.9 (26.1–35.6)	—
β-Cryptoxanthin	0.17 (0.111–0.25)	—
α-Carotene	0.11 (0.07–0.18)	—
β-Carotene	0.278 (0.17–0.43)	—
Lutein	0.22 (0.17–0.28)	—
Lycopene	0.408 (0.23–0.77)	—
Zeaxanthin	0.06 (0.05–0.08)	—
Retinol	1.95 (1.52–2.55)	—

Note: The recruitment of mothers and blood sampling was conducted during the first trimester of pregnancy between 2003 and 2008. —, not applicable; AA, arachidonic acid; ALA, α-linolenic acid; β-HCH, β-hexachlorocyclohexane; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Folate, Folic acid; HCB, hexachlorobenzene; INMA, INfancia y Medio Ambiente–Environment and Childhood (birth cohort); LA, linoleic acid; PCB138, 2,2′,3,4,4′,5′-hexachlorobiphenyl; PCB153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; PCB180, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl; PFHXS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; *p*,*p*′-DDE, dichlorodiphenyldichloroethylene.

Prenatal POPs Determination

Blood samples from mothers were collected at recruitment at the end of the first trimester of gestation (weeks 10–13 of gestation), aliquoted into 1.5 mL cryotubes, and stored at −80°C until their analysis. Concentrations of HCB, β-hexachlorocyclohexane (β-HCH), *p*,*p*′-DDE, and polychlorinated biphenyl (PCB) congeners 138, 153, and 180 were determined in serum samples using gas chromatography (GC) equipped coupled to electron capture detector or mass spectrometer, as previously described.[14,31–33] Concentrations of OCs were adjusted to total serum lipids calculated with the reduced equation using cholesterol and triglycerides determined enzymatically.[34] Concentrations of perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), and perfluorononanoate (PFNA) were determined in plasma samples by column-switching liquid chromatography coupled with tandem mass spectrometry at the Institute for Occupational Medicine, Rhine-Westphalia Technical University (RWTH) Aachen University (Aachen, Germany), as described previously.[24,35] Limits of detection (LODs) ranged between 0.01 and 0.07 ng/mL for OCs and between 0.05 and 0.20 ng/mL for PFAS (Excel Table S1). The coefficients of variation for all PFAS and OC compounds were <15%.[24,31] The selected congeners were detected in all samples included in the present study.

Nutritional Biomarkers Determination

Biomarkers of vitamins, PUFAs, and carotenoids were determined in maternal blood using validated analytical methods described elsewhere.[36–38] Briefly, serum levels of vitamin B12 and folate were measured using a commercially available radioassay.[38] Levels of 25-hydroxyvitamin D3 were measured in plasma by high-performance liquid chromatography (HPLC) using a BIO-RAD kit (Ref. 1956527) (BIO-RAD Laboratories GmbH) as measure of vitamin D status.[37] Concentrations of long-chain PUFAs were determined in maternal plasma using fast-GC.[36] The levels of carotenoids (α- and β-tocopherol, β-cryptoxanthin, α- and β-carotene, lutein, lycopene, zeaxanthin, and retinol) were measured in serum using HPLC with diode array detection and ultraviolet detection at 292 nm in the case of α-tocopherol.[38] All samples included in the study showed levels above the LODs detailed elsewhere (Excel Table S1).[36–38] The biomarkers showed coefficients of variation <10% in interassays and 5% in intra-assays.

Childhood Obesity Outcomes

Trained nurses measured the weight and height of the children at the follow-up visit at 7 years of age [mean ± standard deviation (SD) = 7.7 ± 0.23; ANTIOX data set], using standard protocols. Age- and sex-specific body mass index (BMI) *z*-scores (zBMI) were calculated using the World Health Organization Growth Reference for children 5–19 years of age.[39] Overweight and obesity were defined as the proportion of children with zBMI values of >1 SD and >2 SDs, respectively.[39,40]

Covariates

Information on sociodemographic (age, parity, education) and lifestyle characteristics (e.g., smoking, diet) of the mothers was collected by questionnaires administered to mothers during the first trimester of pregnancy. Measured maternal height and weight reported by the mother at the first trimester visit was used to calculate prepregnancy BMI (in kilograms per meter squared). Data regarding the maternal health status during pregnancy was collected directly from clinical records.

Confounding variables were selected on the basis of *a priori* knowledge supported by published literature on established determinants of maternal levels of POPs, nutritional status/diet, and childhood obesity risk[40–43] and are depicted in a directed acyclic graph (Figure S2). The models for zBMI were thus adjusted for a minimal set of maternal variables at enrollment including prepregnancy BMI (in kilograms per meter squared), age of the mother (in years), education level (primary or without education, secondary, university), region of residence (Gipuzkoa, Sabadell, Valencia) or at delivery [e.g., smoking during pregnancy (nonsmoking, any smoking during pregnancy)]. The models for overweight/obesity were further adjusted for child's sex assigned at birth (female, male) and detailed child age at outcome assessment (in months). Other potential confounders were also tested using the change in estimate method [i.e., change in β or relative risk (RR) ≥ 10%], including parity (categorical: 0, 1, ≥2), maternal physical activity during the first trimester (metabolic equivalent of tasks), supplementation of vitamin B12 during the first trimester (low, medium, and high based on tertiles), adherence to the Mediterranean diet during the first trimester (high, medium, and low adherence; as described by Fernández-Barrés et al.[41]) energy-adjusted dietary intake of key food groups [fish, meat and fruits (in grams per day)] or gestational diabetes risk (diabetes diagnosed previous to pregnancy, gestational diabetes, and none/low risk). In the end, none of those variables were finally retained because of the low impact on our model estimates (i.e., <5% modification of coefficients) and because some of these variables can also be determinants of exposures (e.g., dietary intake of fish) or mediators (e.g., gestational diabetes) instead of confounders.

Data Analysis

The multistep workflow for data analysis is illustrated in Figure S3, covering major questions about the effect of mixtures: Step 1, an exploratory analysis to identify the correlations between POPs and nutrients; Step 2, a preliminary characterization of associations between individual biomarkers and obesity outcomes without accounting for the rest of biomarkers; Step 3, a ranking of biomarker importance accounting for the co-exposure in multipollutant models; Step 4, an estimation of the joint associations between chemical mixtures and obesity outcomes; Step 5, a screening of two-way interactions to select suspected pairs; and Step 6, a refined characterization of those interactions using conventional regression methods to facilitate the interpretation in terms of risk estimation. To this end, we applied a battery of complementary algorithms developed to integrate multiple correlated exposure variables to identify joint effects and interactions.[26,29]

Data preprocessing and unsupervised exploratory analysis. The distribution profiles of POPs and nutrients were explored to characterize the skewness and to identify extreme values, left-censored data, and missing data. Biomarkers with detection frequencies <75% (*p*,*p*′-DDT and PCB118) were removed from the analysis. Participants with nonmeasured exposure data were not considered in the statistical analysis ($n = 153–836$; Figure S1). A multiple multivariate imputation procedure was applied to covariate variables with some missing data following the workflow described elsewhere for data missing at random.[44] Batches of 15 variables were considered in the multivariable imputation models using the R package "mice," developed specifically for each data set. Preprocessing of continuous data included natural log-transformation and scaling to the standard deviation (SD) to improve model fit, if necessary. Exploratory analysis included Spearman's rank correlation analysis to identify correlation patterns between pairs of biomarkers and to support the biomarker grouping in the Bayesian kernel machine regression (BKMR) (see the section "Biomarker importance from multipollutant models" below) and the interpretation of results.

Single-biomarker outcome associations. Multivariate linear and Poisson regression (MLR) with robust variance were used to characterize the associations between individual biomarkers (POPs and nutrients) and continuous (zBMI) or binary outcomes (normal weight vs. overweight and obese combined), respectively. Confounding variables were included in the model as covariates, allowing a flexible estimation of marginal effects. Modified Poisson regression with robust variance was computed using the sandwich approach, which is considered to provide unbiased estimates of risk ratios under potential model misspecification.[45] The method was implemented in R software using the coeftest function with "sandwich" and "lmtest" packages.

Biomarker importance from multipollutant models. Three different statistical methods to examine multipollutant associations were selected based on their complementarity to characterize linear and nonlinear associations, with the capacity to manage confounding data and identify potential interactions. These models also allow the characterization of associations for individual exposures and the identification of interactions (see the section "Joint effects of mixtures"), thus providing a measure of their relative variable importance within each model to rank them while accounting for the rest of biomarkers. A summary of the main features from each model supporting the complementarity is displayed in Excel Table S2.

- **Group Lasso Interaction Network (Glinternet)** is a flexible regularization algorithm designed to identify pairwise interactions in regression models imposing the group lasso (L1) penalties with strong hierarchy.[46] Thus, if an interaction coefficient is estimated to be nonzero, then its two associated main associations also have nonzero estimated coefficients, controlled by the parameter λ. Optimal λ can be selected by cross-validation to identify an adequate bias-variance trade-off. In the present study, 10-fold cross-validation was used to identify the λ exhibiting the lowest mean squared error, computed with the R package "glinternet." To increase the robustness of findings, the models were fitted to 100 bootstrap samples, as described elsewhere.[47] This feature allowed the measurement of coefficient variability and the frequency of interaction detection across bootstrap samples. Averaged model coefficients were used as variable importance scores, considering all of them were at the same scale. The approach is intuitive, may handle a large number of independent variables (e.g., up to 10^5) and their interactions, and is computationally efficient and the results are straightforward to interpret. The method may be limited to characterizing nonlinear associations, and the built-in package does not allow forcing confounding variables out of model penalties.
- **Gradient boosting machine (GBM)** is one of the first approaches proposed to evaluate the joint associations of environmental exposures and their interactions. The use of boosting machines combines a large number of simple regression tree models throughout an iterative process of simpler models' combination (boosting) to improve the overall model fit. We implemented the method using the R packages "gbm" and "dismo."[48] The gbm.step function was computed using the input parameters setup 0.001 for the learning rate and 4 for the tree complexity, the 0.8 bag fraction at 0.8, and a 10-fold cross-validation. The learning rate allows the minimization of the loss function; the tree complexity (interaction depth) controls the number of nodes in the trees, hence the potential interactions; and the bag fraction defines the subsample of data to propose the next step in the tree. To obtain robust estimates, we replicated the model 100 times and extracted the most frequently detected variables (>50%) and their interactions across the replicates. The relative contribution of biomarkers to the overall model fit were used as a variable importance metric. Strengths of GBM includes the capacity to detect nonlinear associations and high-order interactions at a moderate computational cost; however, the interpretability is often challenging, requiring a second modeling step.
- **BKMR.** The BKMR framework is a flexible nonparametric approach that allows the estimation of the overall effect estimate of multiple correlated exposures accounting for confounding variables.[49] The method was implemented with the R package "bkmr" using 10,000 iterations.[50] All variables were included in the model using the variable selection mode, which allows the computation of posterior inclusion probabilities (PIPs) to support the selection of the most relevant variables and to rank the variables according to their probability to be included in the model as an approximate measure of variable importance. Important assets of BKMR compared with the previous methods include the unique capacity to measure the joint effect of the mixture and that the model structure specifically accounts for confounding variables. Although the method can efficiently account for complex interactions, the identification process involves graphical inspection that can become sometimes conflicting.

Joint effects of mixtures. The joint effect of the mixture composed by those POPs and nutrients with higher PIPs was characterized using BKMR with hierarchical variable selection, as described above and using the OverallRiskSummaries function from the "bkmr" R package. The summary estimate displayed the overall effect of biomarkers as the comparison of the predicted outcome when all biomarkers are fixed at given percentiles with the predicted outcome and all biomarkers are fixed at the 10th percentile.

Screening of two-way interactions. In this step, the two-way interactions included in the multipollutant models were identified. With Glinternet, two-way interactions were identified through the inherent strong hierarchical fitting process, together with those main effects likely to be nonzero. In the case of GBM, as other tree-based models, interactions between predictors are inherently included, given that some of the regression trees used are likely to be asymmetric (thus inducing interactions between variables), where the response of one variable depends on the others higher in the tree. The presence of interactions was assessed using the gbm.interactions routine from the "dismo" package. Briefly, the interactions were identified if departures of model predictions for linear combinations of pair of variables was elucidated.[48] For BKMR, two-way interactions were graphically explored using the cross-section plots depicting the exposure–response function for a given exposure when another exposure was fixed at the 25th, 50th, or 75th percentile, fixing the rest of exposures to the median.

To develop a priority list of interactions for a detailed regression analysis, we first selected those chemicals comprising a matching pair of POP and nutrient. In addition, for each model we ranked the pairs based on each specific metric of variable importance as a measure of interaction strength: We used the model contribution for GBM, the standardized model coefficients for Glinternet, and the visual trends of graphical cross-sections for BKMR. For GBM and Glinternet, including bootstrapping, a frequency detection threshold was defined based on the number of interactions identified (e.g., 50%, a large number of interactions; 25%, a low number of interactions). To establish a rank of most influential interactions across three models, we considered the top ranked pairs in at least one method (stronger interactions) or those with lower ranking positions (e.g., weaker interactions) but identified in at least two approaches or obesity outcomes (i.e., continuous or binary).

Characterization of interactions for selected pairs. In the latter step, we aimed to characterize the joint effect of pairs biomarkers (i.e., POPs and nutrients) identified in the previous screening phase to facilitate interpretation. We first built the generalized additive models (GAMs) including the interaction product term for the selected pairs of variables, considering the POPs in continuous scale and nutrients in categorical scale (tertiles), adjusted for the abovementioned covariates. Interaction plots were inspected to characterize the shape of the exposure–response functions and refine the regression models. Unlike distributions of nutrients broken into tertiles for a better interpretation of interactions, POPs were categorized in quartiles if their departure from linearity was graphically visualized. Risk estimates of POPs were then evaluated across the different tertiles of nutrients using linear or robust Poisson regression models for zBMI or overweight/obesity risk, respectively, as detailed above. To formally evaluate the departures from additive joint effects, we also estimated the relative excess risk of overweight/obesity due to interaction (RERI) with the regression models' coefficients from Poisson regression.[28,51] The additive interaction is present when the RERI [95% confidence interval (CI)] is >0 if positive or <0 if negative. All analysis

were conducted with R software (version 4.0.2; R Development Core Team).

Results

Unsupervised Exploratory Analysis

The prevalence of overweight, including obesity, was 43% and 41% in the ANTIOX and PUFA data sets, respectively (Table 2). The respective mean age of children ranged from 7.7 to 7.3 y, and there was an equal proportion of girls and boys in both data sets (Table 2). Mothers had a mean age of 30 y at recruitment with a presence of ∼30% of smokers at some time during pregnancy. Levels of POPs and biomarkers of nutritional status were globally similar between data sets (Table 1) or when compared with those reported in the entire cohort before subsetting (Excel Table S1), yet median concentrations of organochlorine pesticides (OCPs) were slightly higher in the ANTIOX data set compared with the other data sets. The correlation analysis showed high positive correlations ($\rho > 0.5$) between POPs within families (Figure 1A,B; numeric values in Excel Table S3 and S4), and mild or no correlation between families. In turn, *p*,*p*′-DDE was not correlated with PFAS and moderately correlated ($\rho =$ 0.3–0.2) with PCBs, HCB, and β-HCH. Regarding the fatty acids, arachidonic acid (AA) and docosahexaenoic acid (DHA), both showed positive correlations with PCBs and negative correlation with β-HCH ($\rho =$ 0.3 and −0.1, respectively). Similar but weaker association profiles were shown for eicosapentaenoic acid (Figure 1B, Excel Table S4), and linoleic acid (LA) was negatively correlated with PCBs and the rest of the PUFAs. Most OCs and PFAS congeners were not correlated with dietary carotenoids or vitamins, with few exceptions. For instance PFHxS showed mild positive correlations with most nutrients ($\rho =$ 0.1–0.2), whereas *p*,*p*′-DDE was negatively associated with retinol ($\rho =$ −0.3) (Figure 1A, Excel Table S3).

Single-Biomarkers Outcome Associations

The contributions and associations of individual biomarkers with overweight/obesity risk are summarized in Figure 2 (ANTIOX data set) and Figure S4 (PUFA data set), whereas the estimates for zBMI scores can be found in Figures 2 (ANTIOX data set) and 4 (PUFA data set), and numeric results are reported in Excel Table S5. Single-pollutant models showed consistent statistically significant associations of prenatal exposure to HCB and β-HCH with obesity outcomes in children [e.g., adjusted RR = 1.18 (95% CI: 1.07, 1.31) per 1-SD increase in the natural log of HCB (ANTIOX dataset); Figure 3]. PFNA was positively associated with overweight/obesity risk [e.g., RR = 1.10 (95% CI: 1.01, 1.20) per 1-SD increase in in the natural log of PFNA, $p = 0.03$; Figure 3] but not with zBMI (Figures S3 and S5). Null associations were found for the rest of POPs, nonetheless PCB153, PCB180, and PFOA were positively associated with overweight/obesity risk but at limit of conventional statistical significance ($p = 0.06$–0.10; Excel Table S5). Estimates for most nutrients were also null, with the exception of β-cryptoxanthin, zeaxanthin, or α-tocopherol, which were positively associated with zBMI, whereas DHA showed a negative association.

Biomarker Importance from Multipollutant Models

Variable selection methods (Glinternet, GBM, and BKMR) consistently indicated that HCB and vitamin B12 had a high relative importance in the multipollutant models of overweight/obesity risk (Figures 2 and S5) and zBMI (Figures S4 and S6), numeric values are displayed in Excel Tables S6–S11. In turn, β-HCH, PFNA, PFOA, and PFOS also scored high, but the ordering was less consistent across data sets, models, and obesity outcomes. Similarly, the ordering of PUFAs in the importance ranking was less consistent between obesity outcomes. For instance, AA was identified among the most contributing variables on overweight/obesity risk (Figure S5), whereas DHA, LA, and α-linolenic acid (ALA) appeared among the most important contributors on zBMI models (Figure S6). Among the carotenoids, β-cryptoxanthin was the most important biomarker across models and outcomes, followed by α- and γ-tocopherol. In turn, folate showed one of the most inconsistent behaviors across data sets, models, and obesity outcomes, being among the top five PIPs from BKMR in one overweight/obesity model (ANTIOX data set; Figure 2) but scoring low in the rest of the models.

Table 2. Characteristics [mean ± SD or *n* (%)] of mothers and children from INMA Guipuzkoa, Sabadell, and Valencia cohorts (Spain) included in each data set.

Characteristics	ANTIOX $N = 660$	PUFA $N = 558$
Maternal characteristics		
Age at recruitment (y)	31.0 ± 3.8	30.8 ± 3.9
Prepregnancy maternal BMI (kg/m^2)	23.4 ± 4.0	23.7 ± 4.2
Parity		
0	370 (56)	311 (56)
1	250 (38)	214 (38)
≥2	40 (6)	32 (6)
Missing	0	1
Education		
Primary or without education	143 (22)	121 (22)
Secondary	251 (38)	228 (41)
University	265 (40)	209 (37)
Missing	1	0
Region of residence		
Gipuzkoa	272 (41)	141 (25)
Sabadell	—	150 (27)
Valencia	388 (59)	267 (48)
Adherence to Mediterranean diet		
Low adherence	282 (43)	219 (39)
Medium adherence	169 (26)	169 (30)
High adherence	204 (31)	167 (30)
Missing	5	3
Smoking during pregnancy		
No	445 (67)	393 (70)
Yes	206 (31)	157 (28)
Missing	9	8
Child characteristics		
Sex		
Female	325 (49)	277 (50)
Male	335 (51)	281 (50)
Child age (y)	7.7 ± 0.2	7.3 ± 0.6
Child BMI WHO class at 7 y of age		
Thinness (< − SD2; <3rd percentile)	4 (1)	3 (1)
Normal (–SD2 to SD1; 3rd–85th percentile)	371 (56)	325 (58)
Overweight (SD1 to SD2; 85th–97th percentile)	160 (24)	135 (24)
Obesity (>SD2; ≥97th percentile)	125 (19)	95 (17)

Note: The recruitment of mothers was conducted during the first trimester of pregnancy between 2003 and 2008. —, not applicable; BMI, body mass index; INMA, INfancia y Medio Ambiente–Environment and Childhood (birth cohort); SD, standard deviation; WHO, World Health Organization.

Joint Associations of Mixtures

The profile of most relevant chemicals in the mixtures is depicted by the conditional PIPs discussed in the section "Biomarker Importance from Multipollutant Models." The hierarchical variable selection approach, built on groups of biomarker nature (i.e., OCs, PFAS, vitamin, PUFA, and antioxidant), resulted in a balanced composition of top ranked biomarkers with representatives from each family. The overall joint association of the mixture was positive, mostly linear, and significant for overweight/obesity risk

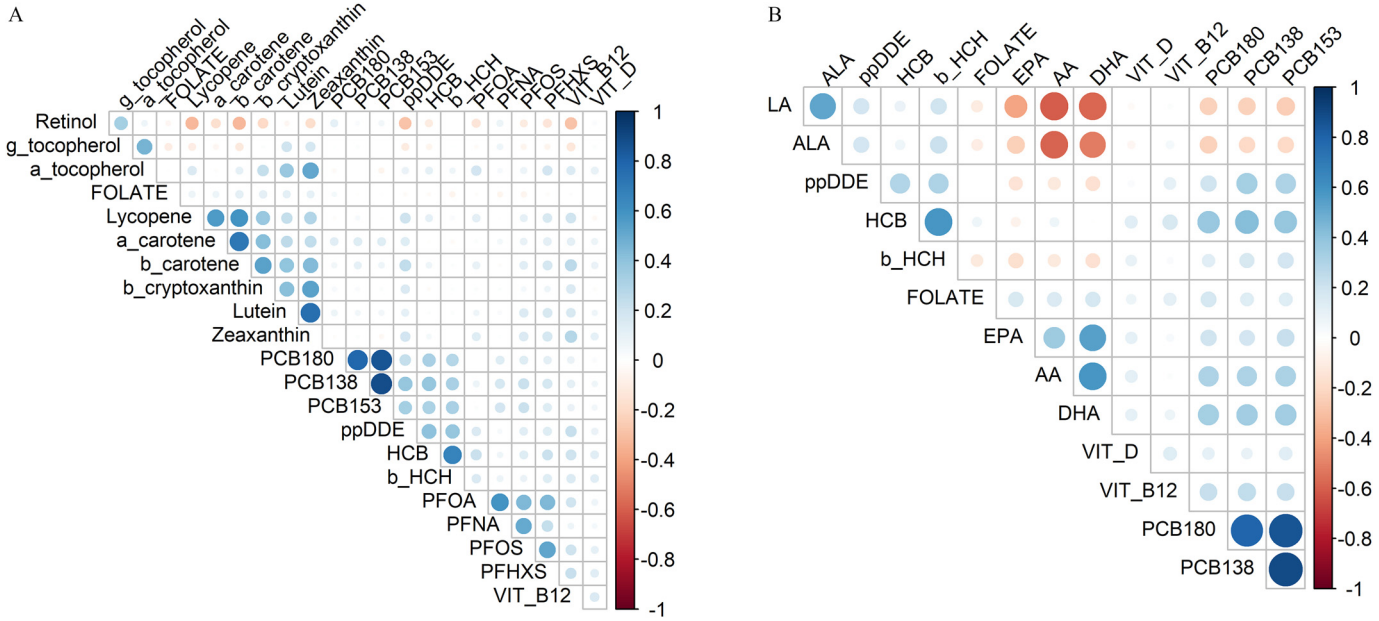


Figure 1. Spearman's correlation plots depicting the association strength between POPs and nutrients in (A) the ANTIOX data set ($n = 660$) and (B) the PUFA data set ($n = 558$). Details of chemical abbreviations are provided in Table 1 and numerical values in Excel Tables S3 and S4. Note: AA, arachidonic acid; ALA, α-linolenic acid; b_HCH, β-hexachlorocyclohexane; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HCB, hexachlorobenzene; LA, linoleic acid; PCB138, 2,2′,3,4,4′,5′-hexachlorobiphenyl; PCB153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; PCB180, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl; PFHXS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; POPs, persistent organic pollutants; *p*,*p*′-DDE, dichlorodiphenyldichloroethylene; VIT_B12, vitamin B12; VIT_D, vitamin D.

(Figure 3A, Excel Table S9) and zBMI (Figure 3B, Excel Table S9). Details of most relevant biomarkers in the mixtures can be found in Figures S11–S14.

Screening of Two-Way Interactions

The screening of interactions relied in three computational methods (Glinternet, GBM, and BKMR) with complementary features to fit the data and identify interactions. Therefore, and to increase the robustness of findings, we selected interactions with the largest strength present in at least one single model or weaker strength but selected by multiple models. Results from each model and outcome are summarized in tables and interaction network plots depicting the bivariate interactions in the ANTIOX data set (Excel Tables S10–S12 and Figures S7 and S8) and the PUFA data set (Excel Tables S10–S12 and Figures S9 and S10). Specific interactions outputs from the GBM model (bootstrap interaction contributions) and Glinternet model (bootstrap interaction coefficients) can be found in Figures S15 and S16 for overweight/obesity risk and in Figures S17 and S18 for zBMI, with the related numerical values in Excel Tables S10 and S11. The frequency of interactions and the variability of strength attributed to each interaction across bootstrap samples is depicted with the raw shaded points and summarized with the means depicted with the white dot.

Overall, we observed larger uncertainty for interaction coefficients in Glinternet models than model contributions from the GBM models. Detection frequency of interactions across bootstrap samples was higher among the findings from PUFA data set (threshold setup 50%) than ANTIOX data set (threshold setup 25%). The detailed graphical output of the bivariate cross-section from BKMR can be found in Figures S11–S14. For the present study, we focused on interactions between POPs and nutrients; however, lists of POP–POP and nutrient–nutrient interactions were also automatically identified from the GBM and Glinternet models and are represented in the interaction network plots (Figures S7–S10). In general, interactions between POPs and nutrients were weak and inconsistent across models, with the presence of more, but weaker, interactions within the ANTIOX data set, whereas in the PUFA data set, we found fewer, but stronger, interactions.

To conduct a more refined analysis, 10 interactions were discerned based on their largest strength/contribution in models or because they were frequently detected across models (highlighted in bold in Excel Table S12), as detailed in the prioritization criteria. For instance, the interaction between HCB and vitamin B12 showed the largest contribution in the GBM models for overweight/obesity risk but that was also graphically suggested in the BKMR model (Figure S11). Similarly, but to lesser extent, the interaction between β-HCH and vitamin B12 was found in the ANTIOX data set (Excel Table S12). The pesticide β-HCH appeared to interact with folate, recurrently detected by GBM in the ANTIOX data set and by Glinternet in the PUFA data set (Excel Table S12). In turn, β-HCH also showed frequently detected interactions with ALA and LA (Excel Table S12). The interaction between PCB138 and LA also exhibited the highest coefficient and largest detection rates in the Glinternet model for overweight/obesity risk. The interactions between PFOA and vitamin B12, and between PFOS and γ-tocopherol, β-cryptoxanthin, and retinol, were also considered of priority interest based on detection frequency and/or strength (Excel Table S12).

Characterization of Interactions for Selected Pairs of POPs and Nutrients

For the selected 10 interactions, we further conducted a regression analysis using GAM models supported by graphical summaries (interaction plots) to identify nonlinearity and the direction of interactions. This visualization allowed, for instance, the identification of the potential synergic effect of HCB and vitamin B12 (Figure 4A) and the potential protective effect of β-cryptoxanthin on PFOS (Figure 4B, with further details in Excel Table S13). To

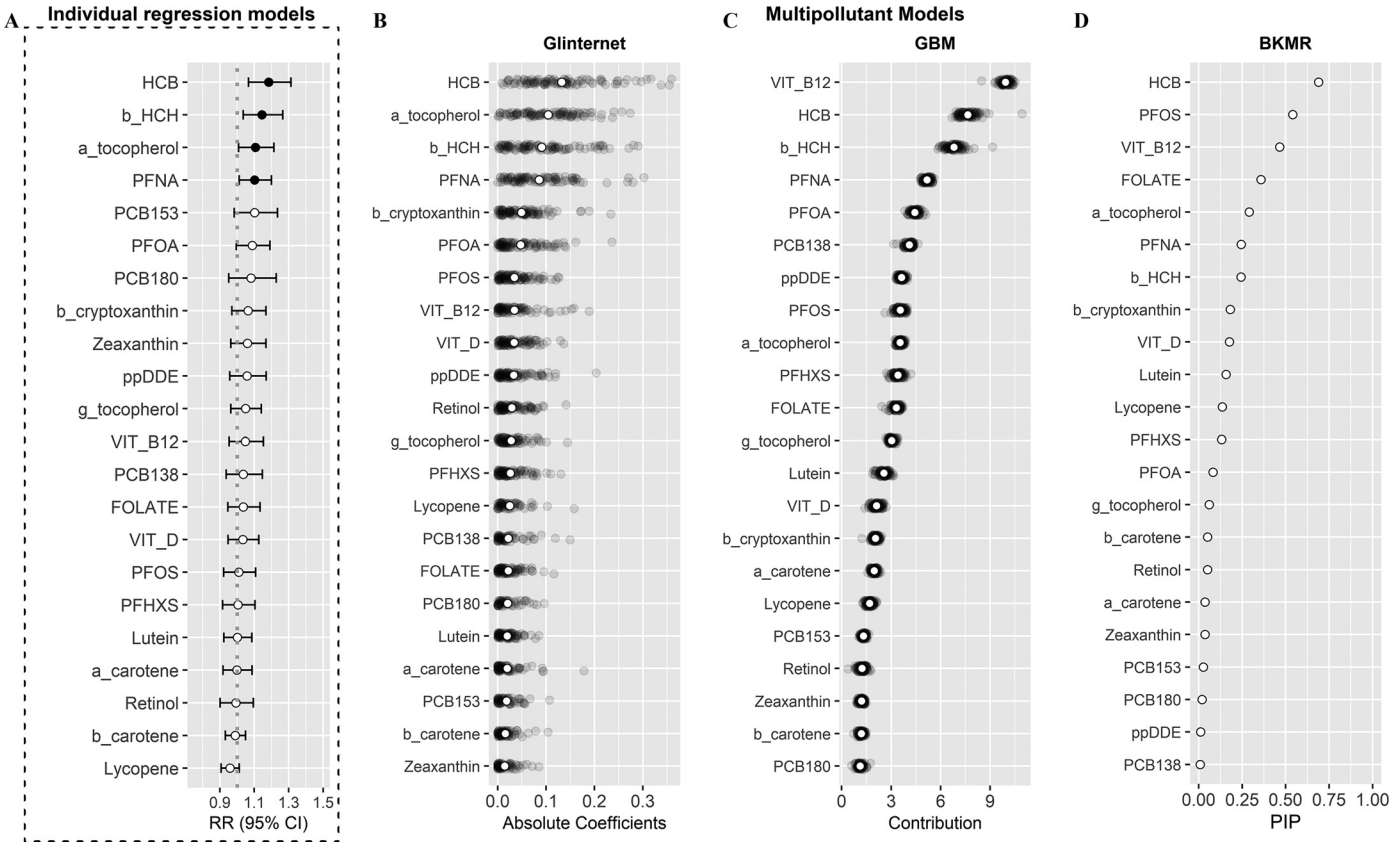


Figure 2. Associations between persistent organic pollutants and nutrients with childhood overweight/obesity within the ANTIOX data set ($n = 660$). (A) Forest plot depicting the associations between individual prenatal exposures (log scaled) and risk of childhood overweight/obesity. Summary estimates from single-biomarker models based on multivariate robust Poisson regression are depicted by adjusted relative risks (RRs) and respective 95% confidence intervals (CIs). Variable importance plots depict the rank of variables based on their relative importance in multipollutant models using the (B) absolute coefficients for Glinternet, (C) model contribution for gradient boosting machine (GBM) regression, and (D) posterior inclusion probabilities (PIPs) for Bayesian kernel machine regression (BKMR). All models were adjusted for maternal age, prepregnancy body mass index, smoking during pregnancy, region of residence, education, child sex, and age. Details of chemical abbreviations are provided in Table 1 and numerical values in Excel Tables S5 and S8. Note: b_HCH, β-hexachlorocyclohexane; HCB, hexachlorobenzene; PCB138, 2,2′,3,4,4′,5′-hexachlorobiphenyl; PCB153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; PCB180, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl; PFHXS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; *p*,*p*′-DDE, dichlorodiphenyldichloroethylene; VIT_B12, vitamin B12; VIT_D, vitamin D.

further characterize the impact of the identified interactions between OCs and vitamins, we used the data set with complete data on OCs and vitamins ($n = 1{,}241$), as depicted in Figure S1.

The detailed regression analysis confirmed those effects: The associations between HCB and childhood overweight/obesity risk and zBMI strengthened at higher levels of vitamin B12 (Table 3). For instance, the associations between HCB and overweight/obesity risk at tertile 2 of vitamin B12 showed an RR = 1.31 (95% CI: 1.11, 1.54), whereas at tertile 1, the associations were close to the null [RR = 0.99 (95% CI: 0.85, 1.14, $p_{Int} = 0.02$)]. Those trends were also observed in the additive scale, with corresponding RERIs = 0.11 (95% CI: 0.02, 0.20) for tertile 2 and 0.12 (95% CI: 0.03, 0.21) for tertile 3. A similar, but weaker, trend was also observed for the interaction between β-HCH and vitamin B12 (Excel Table S14) and between β-HCH and folate (Table 3). In the latter case, despite being statistically not significant at the multiplicative scale, a synergism was suggested in the additive scale between β-HCH and tertile 2 folate with an RERI = 0.11 (95% CI: 0.01, 0.21).

Synergistic interactions were also noticed between PFOS and retinol on the associations with overweight/risk and zBMI, yet at the limit of statistical significance ($p_{Int} = 0.069$) at the multiplicative scale (Table 3). For example, at the highest concentration of retinol, the RR of overweight/obesity of PFOS was 1.42 (95% CI: 0.96, 2.11) for quartile 3 vs. 1. Likewise, in the additive scale the estimates supported the synergism with an RERI = 0.58 (95% CI: 0.11, 1.05) for the same contrast. Similar associations were also observed between PFOS and γ-tocopherol, with an RERI = 0.60 (95% CI: 0.06, 1.15) at the highest levels of PFOS and γ-tocopherol.

In the opposite direction, a negative interaction was observed between PFOS and β-cryptoxanthin. In this case, the associations between PFOS and overweight/obesity or zBMI, were substantially higher at the lowest tertile of β-cryptoxanthin, reaching an RR = 1.59 (95% CI: 1.02, 2.05) for quartile 4 vs. 1 of PFOS. Although the interaction was not statistically significant in the multiplicative scale ($p_{Int} > 0.1$), the RERIs supported antagonisms in the additive scale (Table 3). Finally, the synergistic interactions suggested between POPs and PUFAs appeared to be mostly weak and nonstatistically significant in both scales (Excel Tables S14 and S15) with the exception of PCB138 and LA, which showed an RERI = 0.18 (95% CI: 0.02, 0.33) in the third tertile.

Discussion

In the present study, we attempted to develop and apply a comprehensive statistical framework to evaluate the mixture effect of

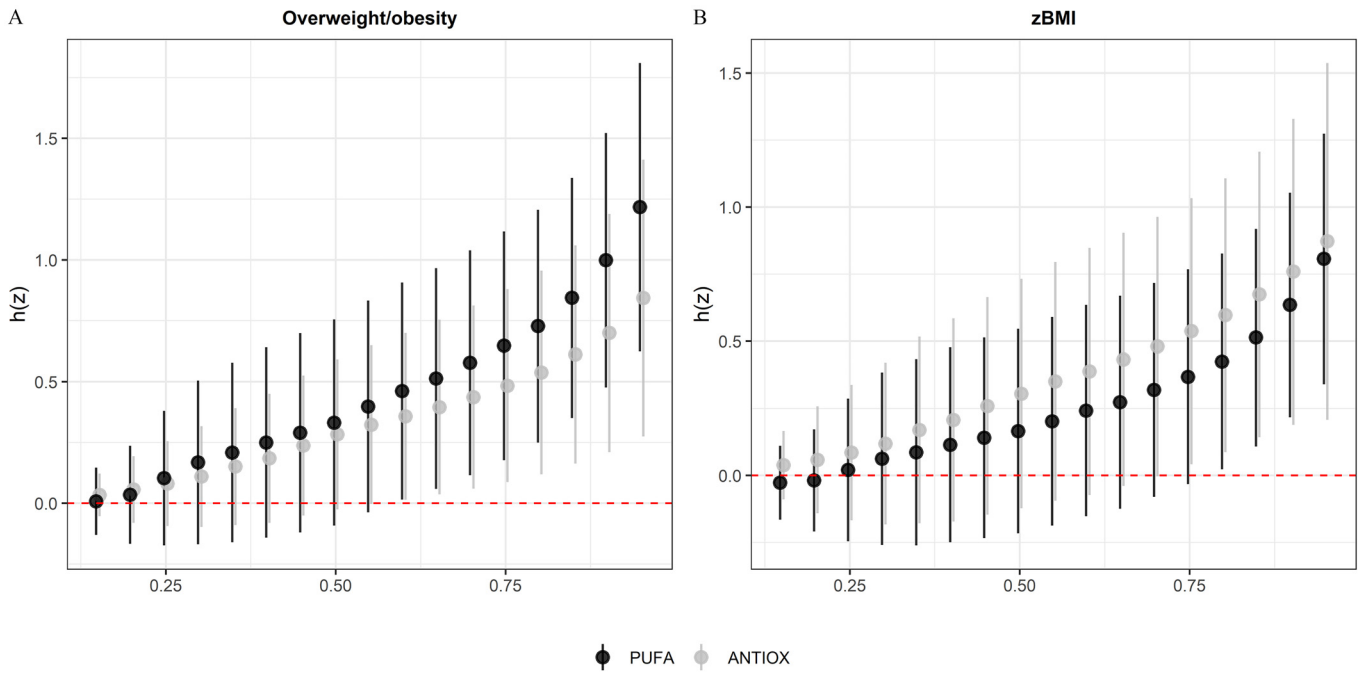


Figure 3. Overall effect estimates from Bayesian kernel machine regression (BKMR) on the association between mixtures of POPs and nutrients and (A) childhood overweight/obesity risk and (B) body mass index *z*-score (zBMI) for the mixtures of chemicals selected with the hierarchical procedure from ANTIOX data set (gray) and the PUFA data set (black). Details of most relevant chemicals in the mixtures are depicted in Figures S10–S13. Graphs show the difference in the effect estimates when all exposures are at a particular quantile compared with when all are at the 10th quantile as reference. All models were adjusted for maternal age, prepregnancy body mass index, smoking during pregnancy, and region of residence and education; child sex and age were also included in overweight/obesity models. Numerical values can be found in Excel Table S9.

prenatal exposure to POPs and nutrients on childhood overweight/obesity. This approach, applied to the population-based birth cohort study INMA, confirmed findings from previous studies in this cohort, highlighting the role of prenatal exposure to HCB[5,14,22] and β-HCH[22] on childhood obesity risk and providing evidence of a positive joint effect of the mixture of POPs and nutrients. Screening for interactions using advanced approaches highlighted a number of potential combinations. Conventional regression models allowed translating those interactions into more meaningful effect estimates in terms of inferential interpretation. Among the 10 POPs–nutrient interactions retained in the screening step, HCB–vitamin B12 was the most consistent across models

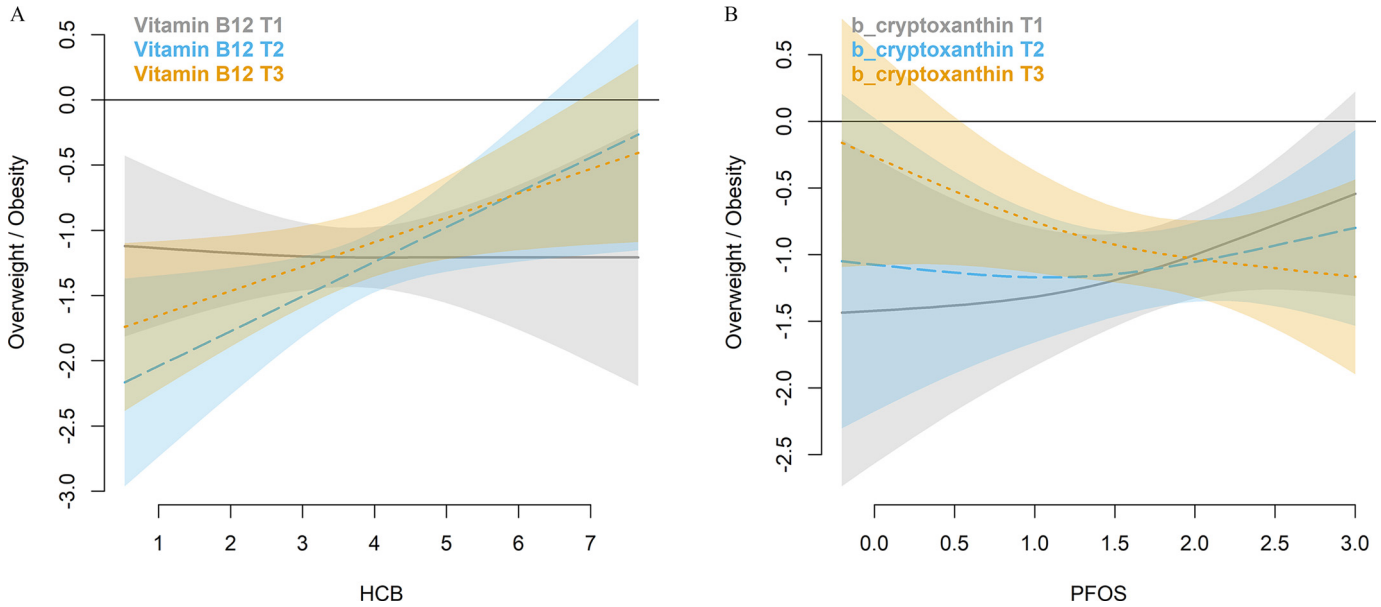


Figure 4. Interaction plots on the associations between (A) hexachlorobenzene (HCB, log increase) and tertiles of vitamin B12 and between (B) perfluorooctane sulfonate (PFOS, log increase) and tertiles of β-cryptoxanthin on overweight/obesity risk. Tertile 1 (T1) is depicted by a solid line; tertile 2 (T2) by a long-dashed line, and tertile 3 (T3), by a dotted line. All models were adjusted for maternal age, prepregnancy body mass index, smoking during pregnancy, region of residence, education, and child sex and age. Coefficient details can be found in Excel Table S13.

Table 3. Summary estimates for the associations between selected POPs across tertiles of nutrients with obesity outcomes from Poisson/lineal regression models with a cross-product interaction term.

			Overweight/obesity					zBMI		
POP	Nutrient	Tertile	RR	95% CI	p_{Int}	RERI	95% CI	β	95% CI	p_{Int}
HCB (log)[a]	Vitamin B12 (pmol/L)	<302	0.99	(0.85, 1.14)	0.022	Ref		−0.07	(−0.21, 0.07)	0.002
		302–339	1.31	(1.11, 1.54)		0.11	(0.02, 0.20)	0.26	(0.12, 0.40)	
		>339	1.21	(1.06, 1.37)		0.12	(0.03, 0.21)	0.16	(0.05, 0.28)	
β-HCH (log)[a]	Folate (mmol/dL)	<11.4	1.00	(0.88, 1.15)	0.260	Ref		0.00	(−0.12, 0.12)	0.110
		11.4–19.0	1.16	(1.02, 1.32)		0.11	(0.01, 0.21)	0.16	(0.06, 0.27)	
		>19.0	1.13	(1.02, 1.25)		0.09	(−0.02, 0.20)	0.12	(0.03, 0.21)	
PFOS[b]										
Q2	Retinol (mmol/L)	<1.7	0.90	(0.59, 1.38)	0.069	Ref		−0.34	(−0.80, 0.13)	0.076
Q3			0.77	(0.48, 1.22)		Ref		−0.26	(−0.73, 0.20)	
Q4			0.97	(0.63, 1.49)		Ref		−0.13	(−0.59, 0.33)	
PFOS										
Q2	Retinol (mmol/L)	1.7–2.3	1.20	(0.83, 1.74)		0.12	(−0.23, 0.81)	0.37	(−0.05, 0.78)	
Q3			1.09	(0.74, 1.61)		0.32	(−0.19, 0.84)	0.26	(−0.17, 0.69)	
Q4			1.16	(0.79, 1.69)		0.18	(−0.36, 0.72)	0.23	(−0.19, 0.64)	
PFOS										
Q2	Retinol (mmol/L)	>2.3	0.62	(0.35, 1.10)		0.12	(−0.80, 0.38)	−0.30	(−0.72, 0.12)	
Q3			1.42	(0.96, 2.11)		0.58	(0.11, 1.05)	0.35	(−0.06, 0.76)	
Q4			1.10	(0.69, 1.74)		0.11	(−0.45, 0.67)	0.07	(−0.35, 0.49)	
PFOS[b]										
Q2	β-cryptoxanthin (mmol/L)	<0.1	1.32	(0.82, 2.11)	0.244	Ref		0.11	(−0.30, 0.52)	0.367
Q3			1.34	(0.85, 2.13)		Ref		0.38	(−0.07, 0.82)	
Q4			1.59	(1.02, 2.50)		Ref		0.26	(−0.17, 0.69)	
PFOS										
Q2	β-cryptoxanthin (μmol/L)	0.1–0.2	0.79	(0.49, 1.28)		0.12	(−1.48, 0.30)	−0.02	(−0.45, 0.41)	
Q3			1.05	(0.70, 1.58)		−0.28	(−1.09, 0.54)	0.12	(−0.31, 0.55)	
Q4			1.12	(0.74, 1.69)		−0.44	(−1.32, 0.45)	0.28	(−0.14, 0.70)	
PFOS										
Q2	β-cryptoxanthin (mmol/L)	>0.2	0.75	(0.50, 1.11)		0.12	(−1.75, 0.20)	−0.32	(−0.77, 0.12)	
Q3			0.86	(0.60, 1.23)		−0.59	(−1.50, 0.32)	−0.19	(−0.61, 0.24)	
Q4			0.71	(0.47, 1.07)		−1.12	(−2.19, −0.05)	−0.35	(−0.79, 0.09)	
PCB138 (log)[c]	Linoleic acid	<27.6	0.96	(0.75, 1.24)	0.297	Ref		−0.09	(−0.35, 0.16)	0.372
	(percentage fatty acids)	27.6–33.4	1.10	(0.86, 1.41)		0.10	(−0.13, 0.33)	0.03	(−0.21, 0.27)	
		>33.4	1.25	(0.99, 1.58)		0.18	(0.02, 0.33)	0.14	(−0.08, 0.36)	

Note: Additive interactions on overweight/obesity risk are depicted by the RERI and respective 95% 95% CI. All models were adjusted for maternal age, prepregnancy body mass index, smoking during pregnancy, education and region of residence, in addition models on overweight/obesity risk were further adjusted for child sex and age. Detailed levels of POP quartiles are displayed in Excel Table S15. Details of chemical abbreviations are provided in Table 1. CI, confidence interval; HCB, hexachlorobenzene; p_{Int}, *p*-Value from interaction testing PCB138, 2,2′,3,4,4′,5′-hexachlorobiphenyl; PFOS, perfluorooctane sulfonate; POP, persistent organic pollutant; Q, quartile; Ref, reference; RERI, relative excess risk due to interaction; RR, adjusted relative risks; zBMI, child body mass index *z*-score; β-HCH, β-hexachlorocyclohexane.

[a]Population sample size $n = 1{,}241$ (see details in the study flowchart in Figure S1).

[b]Population sample size $n = 660$ (ANTIOX data set).

[c]Population sample size $n = 558$ (PUFA data set).

and outcomes, and the regression models suggested a potential synergistic effect. Interaction between PFOS and β-cryptoxanthin suggested a protective effect of the antioxidant on overweight/obesity risk, with a higher risk associated with PFOS exposure being observed only at the lower concentrations of β-cryptoxanthin.

Despite the rise of multipollutant modeling approaches, few studies have considered mixtures of biomarkers others than pollutants.[52] In fact, some dietary nutrients have the potential to counterbalance the effects of environmental pollutants, highlighting the interest of accounting for them in the mixture model.[18,53] The mixture analysis allowed the identification of an unexpected synergistic effect of vitamin B12, strengthening the associations of HCB. Vitamin B12 is an essential hydrophilic vitamin found mainly in animal food products, with dairy products and meat the major contributors and exhibiting short half-lives in the body.[54] Despite the lack of international consensus on the optimal levels during pregnancy, there is some agreement that the range between 220 and 850 pmol/L would be adequate.[55] Thus, considering that median levels of vitamin B12 within our population were 350 pmol/L (interquartile range: 279–435 pmol/L) most of our study population would fall within that optimal range, with only 12 participants exceeding the upper threshold. Considering that sources of vitamin B12 in humans are exogenous, the blood levels may be determined either by the dietary intake or as result of their metabolism. There is the possibility that vitamin B12 may be confounding the true effect of some other unmeasured concomitant nutrients or specific food habits associated with HCB[56] or childhood obesity[41,57]; however, adjustment for meat intake did not modify our results, consistent with previous findings.[42,43] Our secondary analysis showed that vitamin B12 levels were statistically associated with vitamin supplementation and meat intake, hence supporting the role of intake in the vitamin status, which has been also reported in the same cohort.[58] Mechanisms supporting the joint effect of OCs and vitamin B12 or folate on overweight risk can involve epigenetic programming.[59,60] A maternal intake of methyl-group donors (e.g., folates, vitamin B12) could also alter the DNA methylation profiles of an offspring's metabolic genes.[61] In turn, HCB and vitamin B12 can both individually impact the metabolic programing of adipocytes during differentiation or their DNA methylation profiles.[62,63] Indeed, vitamin B12 plays a crucial role in humans as a cofactor of methionine synthase, which is actively involved in methionine biosynthesis via the remethylation of total homocysteine. Interestingly, two enzymes involved in *S*-adenosyl methionine synthesis (phosphatidylethanolamine *N*-methyltransferase and glycine *N*-methyltransferase), are transcriptional targets of the aryl hydrocarbon receptor,[64] which is activated by HCB.[65] Thus, we may hypothesize that a maternal intake of methyl-group donors

(i.e., vitamin B12) together with a higher HCB exposure contribute to an increased lipogenesis. Considering the active research to establish more accurate recommendations and thresholds of vitamin supplementation during pregnancy,[66] future studies should consider the concomitant presence of environmental pollutants.

The protective effect of β-cryptoxanthin on the association between PFOS and childhood obesity also deserve attention. β-Cryptoxanthin is a naturally occurring carotenoid found in many foods of plant and animal origin (e.g., oranges, apples, egg yolk). It is closely related to β-carotene and has antioxidant properties. Conversely, PFOS is known for its prooxidative activity[67] and increase of adipogenesis *in vitro*,[68] but epidemiological studies are globally inconsistent. A study in the same INMA cohort showed mild or null associations,[24] whereas other previous studies have generally shown inconsistent findings,[69] and some authors have even proposed PFOS as an anti-obesogen.[70] For the first time, we were able to observe a higher risk of obesity among children exposed to higher levels of PFOS and lower levels of this antioxidant during gestation. Current evidence with adult women has shown that concentrations of carotenoids, including β-cryptoxanthin, are inversely associated with BMI and waist circumference, with major effect modification by exposure to toxicants, such as by smoking.[71] In experimental studies, β-cryptoxanthin exerted an anti-obesogenic effect, reducing the body fat of mice and increasing the expression of uncoupling protein 1 (UCP1) in adipose tissue via the retinoic acid receptor (RAR).[72] In turn, PFOA and PFOS have also been shown to activate UCP1 in brown adipose tissue, which can modulate the food intake and body weight[70]; however, our findings suggest the presence of other potential mechanisms to explain the obesogenic effects of PFOS. For instance, PFOS and PFOA are activators of the peroxisome proliferator-activated receptor-alpha (PPAR-α) in human cells.[73] PPAR-α and RAR share a common dimerization partner, the retinoid X receptor (RXR).[74] Hence, the activation of both receptors (PPAR-α by PFOS) and (RAR by β-cryptoxanthin) could lead to a competitive effect toward this RXR partner.[75]

Biomonitoring studies during perinatal periods supports the fact that women are exposed to multiple environmental chemicals during pregnancy and lactation periods, critical windows for offspring development.[53,76] This exposure paradigm has stimulated the increasing interest in characterizing the joint effect of environmental chemicals during pregnancy on an offspring's health outcomes, raising the development and implementation of statistical approaches to address mixture related questions.[29,52] Although the list of algorithms and statistical methods has been growing during the last few years, no one specific method consistently outperforms the others as assessed in simulation studies.[26,29,77] Therefore, we conceived an approach combining multiple models to strengthen the robustness of our findings supported by the specific features from each algorithm. The method selection included Glinternet, GBM, and BKMR, based on previous literature supporting their relatively high statistical performance and capacity to characterize the joint associations of correlated variables accounting for their interactions.[26,78] A simulation study showed that BKMR, in the case of nonmonotonic exposure–response relationships, may outperform penalized regression methods that assume linearity,[29] a group of methods which includes the Glinternet model. Identifying relevant components of the mixtures remains a major question in terms of public health but also in terms of regulatory decision-making. Statistically, this is commonly accomplished by using variable selection, a process that becomes especially challenging as correlation between variables increases.[79] Although data-driven approaches such as BKMR may improve the predictive performance of models, those may fail to attribute the true effect to the right candidates within the correlated cluster.[27] This fact, together with the different nature of variable selection method (e.g., Glinternet and GBM), may help to explain some inconsistencies in the variable importance rankings between models. In any case, the variable selection process prevents selection of highly correlated variables in the model and, thus, reduces the risk of amplification bias expected in chemical mixtures.[80] An alternative way to leverage this issue would be to use *a priori* toxicological knowledge to inform the variable selection process, especially in exploratory contexts where improving predictive performance falls out of scope. Selection of relevant interactions follows a process similar to that of the main effects, based on likelihood penalization in the case of Glinternet or spike-and-slab priors in the case of BKMR.[46,49] We noticed that detection of interactions becomes specially challenging and inconsistent between models when the interaction is weak,[81] something supported by the fact that low-powered studies are prone to false positive detection.[82] Thus, the agreement criteria across screening methods could be a solution to attenuate the risk of false positives, as observed in the case of the strongest interaction between HCB and vitamin B12. To increase the robustness of findings from the Glinternet and GBM models, we applied a bootstrapping approach with 100 replicates, allowing the identification of interactions in terms of frequency of detection and relative contribution or strength. An additional factor affecting those inconsistencies may also be related to the differences on chemical distributions and related mixture structures between data sets. For instance, we noticed slightly higher median levels of OCPs in the ANTIOX data set compared with the rest of the data sets (Excel Table S1), consequently impacting the data set specific risk estimates. The findings also highlighted the presence of other interactions (i.e., pollutant–pollutant or nutrient–nutrient) not discussed in the present manuscript, but which may help to illustrate the complex interplay of chemicals within the mixture.

The present study should be considered with caution in light of some study limitations. First, the sample size was relatively small ($n = 558$–$1{,}241$) for the exploration and characterization of interactions and might have resulted in low power to detect interacting effects and thus could be prone to false negatives. Second, we applied a data-driven approach to explore potential interactions with biological meaning. The high correlation between some pollutants and the current lack of congener-specific knowledge about their obesogenic potential, may increase the risk of exposure misclassification, thus attributing the interactive effect to the wrong chemical within the clusters of highly correlated variables. Current *in vitro* and *in vivo* studies about obesogenic effects of POPs are relatively limited to a few congeners, which in turn, can be highly correlated in biological matrices. For simplicity and due to the limited sample size, we focused the study to characterize two-way interactions; however, higher-order interactions cannot be neglected, either between pollutants and nutrients but also, with other individual characteristics such as maternal smoking or child's sex, as previously observed.[83] We may have also failed to accurately measure vitamin levels representative of all pregnancy given that these nutrient biomarkers reflect the current intakes or their relatively short timeframes due to their short half-lives, which range from several minutes in the case of vitamin B12 (~ 7 min) to days (26–76 d) for carotenoids and even longer for folate.[84,85] Nevertheless, we believe that measurement error would be homogeneously distributed across the study population; hence, it is expected that it would lead to null findings instead of spurious associations. In addition, the biomarkers used have been positively associated with established predictors from food frequency questionnaires administered to pregnant women.[38] In the case of POPs, a single spot blood measurement is often considered indicative of long-term exposure given their long elimination half-lives as also shown in the

INMA cohort.[35,86] The statistical framework was developed assuming interactions between nutrients and POPs; however, the exploratory nature of this study did not have the ambition of elucidating the detailed underlying causal structure to determine, for instance, whether nutrients were actually effect modifiers.[87] In this regard, additional causal structures could also be considered in the light of potential toxicokinetic effects of the considered nutrients on the biomarker levels of POPs. For instance, it could be plausible that reported anti-obesogenic effects of certain carotenoids may interfere with the circulating levels of POPs,[19] hence contributing to measurement error and amplification bias. In addition, residual confounding cannot be completely ruled out, especially in the light of false negatives, despite the sensitivity analysis showing that our findings are robust to an extensive list of potential confounders reflecting dietary habits and lifestyle. The exploration of interactions is an emerging and active field of methodological research, and other novel approaches accommodating the complexities of pollutant data sets could be considered in future studies.[88,89] Finally, the biological interpretation of statistical interactions requires some attention, in part because of the different implication of interaction scales or the definitions used in different fields.[90] Following current recommendations, we reported the interactions in multiplicative and additive scale,[28] and we believe that our findings may help to develop biological hypothesis for future toxicological studies and better interpret inconsistent findings in epidemiological studies.

In summary, the present study supports the hypothesis that nutritional status during pregnancy can modify the effect of environmental pollutants on child health. Specifically, we found that high levels of vitamin B12 may strengthen the associations between prenatal exposure to HCB and childhood obesity. In the opposite direction, the dietary antioxidant β-cryptoxanthin might have a protective effect against the obesogenic effects of PFOS. Our findings suggest that independent models may fail to identify weak interactions between pollutants and nutrients; therefore, combining complementary models may be a more powerful approach to consider. In the light of the public health implications of these findings, further observational and experimental research will be required for confirmation of these findings and gaining insight into the complex interplay between pollutants and nutrients during pregnancy on the metabolic programing of the offspring. As highlighted, these interactions may uncover subpopulations at risk for specific chemicals under regulatory policies, but they also support the need for more accurate nutritional guidelines during pregnancy.

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Associations between internal concentrations of fluorinated and organochlorinated chemicals in women and *in vitro* fertilization outcomes: A multi-pollutant study☆

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ABSTRACT

The impact of persistent organic pollutants (POPs) on reproductive health is still poorly understood, even though infertility management has high associated societal and economical costs. The aims of this study were to characterize the internal levels of polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and perfluoroalkylated substances (PFAS) in women undergoing *in vitro* fertilization (IVF); and evaluate their association with IVF outcomes, individually and as mixtures in a combined multipollutant approach. Thus, 136 women undergoing IVF treatment at Nantes University Hospital (France) were prospectively recruited between 2019 and 2020. Serum samples were analyzed using liquid chromatography with tandem-mass spectrometry for 14 PFAS. Follicular fluid was analyzed with gas chromatography coupled to high resolution mass spectrometry for 14 PCBs and 25 OCPs. Intermediate and clinical IVF outcomes were ascertained by embryologists and clinicians using standardized protocols. Multivariate Poisson regression models and Bayesian Kernel Machine Regressions (BKMR) were used to identify individual and joint associations between POPs and IVF outcomes adjusting for age, body mass index (BMI) and anti-Müllerian hormone. The results showed that most POPs were widely present in women, and globally not associated with clinically relevant IVF outcomes, like live birth rates. Nonetheless, negative associations between PCB138 and trans-nonachlor with useable blastocysts were identified, β −0.28 (95%CI [-0.52; −0.04] $p = 0.02$) and β −0.22 (95%CI [-0.40; −0.03] $p = 0.02$). Conversely, PCB28 showed positive associations with the number of useable blastocysts, pregnancy rate and live birth rate. The BKMR analysis suggested the lack of association of the mixture with intermediate and clinical outcomes. The study supports the need of conducting further studies in a larger population sample in order to ensure sufficient statistical power to identify modest effects and a robust stratification analysis to account for the large underlying disease heterogeneity.

1. Introduction

Infertility, defined by the absence of pregnancy after 12 months or more with regular unprotected sexual intercourse (World Health Organization, 2018), affects 8–12% of reproductive aged women worldwide (Vander Borght and Wyns, 2018) or one out of 6 couples (Datta et al., 2016). It is estimated that 2–6% of all births are conceived through Assisted Reproductive Technology (ART) (Pinborg, 2019). Infertility management has associated high societal and economical costs, hence, it has become a public health priority worldwide (Barriere et al., 2018).

A number of life-style and individual factors have been identified as modulators of *in vitro* fertilization (IVF) success, including age, body mass index, nutrition or smoking (Howell et al., 2020). In addition, there is increasing concern amongst medical community and policy makers

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over the impact of environmental pollutants on human health, with growing evidence on their deleterious effects on reproductive health (McCue and Denicola, 2019; Thalia et al., 2019). For instance, the available evidence suggests that female exposure to persistent organic pollutants (POPs) may reduce fecundability (Kahn et al., 2021) or be associated with the pathogenesis of some reproductive disorder such as endometriosis for instance (Matta et al., 2019).

POPs includes legacy pollutants like organochlorine pesticides (OCP) used in agriculture or polychlorinated biphenyls (PCBs) in many industrial applications (Yang et al., 2021). There are also more emerging synthetic chemicals including the vast family poly- and perfluoroalkylated substances (PFAS). These chemicals are widely used in the production of consumer products due to their amphiphilic properties (Sunderland et al., 2019). The physical and chemical stability and the lipophilic character of POPs are responsible for their bioaccumulation in biota and their biomagnification in food chain, with the potential to reach humans and accumulate in their fat during the lifetime, despite the severe international actions to ban or regulations several decades ago (Domingo, 2012; Porta et al., 2012; Van den Berg et al., 2017).

Some POPs still represent a concern for human health despite the declining trends of exposure, mainly because of their ability to interfere with multiple signalling pathways and biological functions at very low concentrations. Concerning reproductive function, POPs like PCBs have been associated with impaired fertility in animals (He et al., 2021) and decreased spontaneous fertility among humans (Buck Louis, 2014; Kahn et al., 2021). Recent studies showed that a number of POPs from various families are detected in follicular fluid and serum of women undergoing ART treatments, raising concern about their deleterious effects on IVF outcomes (Björvang and Damdimopoulou, 2020; Cabry et al., 2020; Lefebvre et al., 2021). Some PCBs and OCPs may also impair IVF outcomes such as embryo quality and pregnancy rates (Younglai et al., 2002; Bloom et al., 2017; Petro et al., 2012; Meeker et al., 2011; Björvang et al., 2021). Nevertheless, the evidence remains inconclusive (Al Hussaini et al., 2018; Jirsova et al., 2010; Weiss et al., 2006). Indeed, some sources of heterogeneity and research gaps have been identified in a recent systematic review on the associations between POPs and IVF outcomes (Lefebvre et al., 2021). For instance, many studies have been conducted on a very limited number of samples ($n < 30$), often with poor control of confounding factors and failing to address the exposure to chemicals as mixtures (Lefebvre et al., 2021). Additionally, no studies in the topic have been conducted in France to date.

Thus, the aims of this study were to 1) characterize levels of PCBs, OCPs and PFAS in serum or follicular fluid from women undergoing IVF; and 2) evaluate their association with IVF outcomes, individually and as mixtures in a combined multipollutant approach.

2. Materials and methods

2.1. Population

Women aged 18–43 years, referred for IVF to the ART centre of the University Hospital of Nantes, France, were prospectively recruited between June 2019 and June 2020. Approximatively 85% of eligible women were finally included. IVF outcomes did not differ between participants and non-participants over the study period (data not shown). This analysis includes 136 women undergoing IVF for various reasons, either female infertility (i.e. endometriosis, polycystic ovarian syndrome [PCOS], premature ovarian insufficiency, tubal infertility, unexplained infertility), or non-female factor infertility (i.e. male infertility or egg donation). Only one IVF cycle per woman was considered. Cancelled cycles and preimplantation genetic testing (PGT) cycles were excluded. Detailed study workflow is depicted in Fig. S1.

During the first visit, patients were interviewed to collect demographic and lifestyle information (menstrual history, smoking habits, dietary habits, occupational and residential history, level of education, weight variations and physical activity). Physical activity has been defined according to World Health Organization (WHO) recommendations, i.e. an adult should do 150 min of moderate activity per week. We considered 3 groups: 1)"none": women with no physical activity, 2)"moderate": women respecting the WHO recommendations and 3) "high": women practicing a higher activity in time or intensity. Clinical data (age, body mass index [BMI], infertility etiology, previous pregnancy history, medical and surgical history, medication exposure, history of ART), ovarian stimulation parameters and IVF outcomes were anonymously collected from the hospital database, in accordance with the French National Commission for information and Liberties (CNIL). Research protocol was approved by the local Ethics Committee (GNEDS) on 11th of June of 2019. Written informed consent was obtained from all women for all participants.

2.2. IVF cycles

All patients underwent controlled ovarian hyperstimulation (COH) with antagonist protocol with Gonadotropin-Releasing Hormone (GnRH). Serum estradiol and progesterone levels, follicular development and endometrial thickness were monitored according to standard protocol. Ovulation was triggered when at least 3 follicles reached $\geq$17 mm diameter with either recombinant hCG (Ovitrelle®, Merck-Serono®, France) or GnRH agonist (Decapeptyl®, Ipsen®, France) according to the risk of Ovarian HyperStimulation Syndrom.

Oocyte retrieval was performed 36 h later. ICSI (Intra-cytoplasmic spermatozoid injection) or conventional IVF was performed according to sperm quality and couple's history. Oocyte maturity rate was calculated as the number of mature oocyte over the total number of oocyte retrieved. Fertilization rate was calculated as the number of normally fertilized zygotes over the number of MII oocytes injected.

Embryos were then systematically cultured at 37 °C up to the blastocyst stage in single-step culture medium (G-TL®, Vitrolife®, Sweden) under low oxygen atmosphere (6% CO2, 5% O2). Blastocysts were evaluated according to Gardner and Schoolcraft classification on day 5 and/or day 6. On day 5, all blastocysts (early cavitating B1 to hatched B6) could be considered useable for transfer or freezing, except those with grade C trophectoderm. When no blastocyst was available on day 5, culture was prolonged up to day 6, where only blastocysts $\geq$ B3BB could be considered for transfer or freezing. D5BUR (day 5 useable blastocyst rate) was calculated as the number of blastocysts compatible with transfer or vitrification over the number of fertilized oocytes (Hammond, 2019). Vitrification was performed with VitBlast® kit (Vitrolife®, Goteborg, Sweden) according to the manufacturer's instructions.

When a least one blastocyst was transferred, a pregnancy test was performed 11 days after embryo transfer. Clinical pregnancy (CP) was defined as the presence of an intrauterine gestational sac with fetal heart activity at 4–6 gestational weeks, confirmed by ultrasound examination. Live birth was defined as the birth of a newborn beyond 22 weeks of gestation. Live birth rate (LBR) was calculated as the number of deliveries over the number of first embryo transfers (either fresh or frozen-thawed). Cumulative live birth rate (CLBR) was calculated as the total number of live births per ovum pickup, including all subsequent embryo transfers, either fresh and frozen-thawed.

2.3. Sampling and laboratory analysis of POPs

Blood samples were collected 1–2 days before the start of COH, and serum (5 ml) was frozen and stored at −80° until analysis. Follicular fluid samples (10 ml) were collected and frozen at −80 °C the oocyte retrieval day. Constrained by the biocollection, the determination PCBs and OCPs was conducted in serum and PFAS in follicular fluid, supported by the high correlation of compounds between these matrices (Lefebvre et al., 2021).

All analyses were performed under blinding conditions (i.e. analysts were not informed about the outcome status) and following ISO17025:2005 accredited methods. Briefly, 14 PFAs were measured in

serum using liquid chromatography with tandem-mass spectrometry (LC-MS/MS) as previously detailed (Mancini et al., 2020). Serum samples first underwent an alkaline digestion followed by a two-stage solid-phase extraction and purification using polymeric Oasis® HLB and graphitised carbon (ENVI-Carb®) cartridges. PCBs and OCPs were measured in follicular fluid using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS), slightly adapting the methodology previously detailed for serum (Koual et al., 2019; Matta et al., 2022). Serum lipid content was determined with enzymatic kits (Biolabo, Maizy, France), and calculated as the sum of phospholipids, triglycerides, total cholesterol and free cholesterol. Concentrations below the limit of detection (LOD) were assigned a value of LOD/2.

2.4. Statistical analysis

We first examined individual and demographic characteristics (age, BMI, education, living environment, smoking status, ovarian reserve, type and duration of infertility) using frequencies, percentages or mean ±standard deviation (SD). Then, ovarian stimulation (IVF cycle rank, COH duration and total dose of gonadotropins), intermediate outcomes (number of oocytes retrieved, oocyte maturity rate, fertilization rate, number of useable blastocyst, D5BUR), as well as clinical IVF outcomes (implantation rate, pregnancy rate, clinical pregnancy rate, live birth rate after 1st blastocyst transfer and cumulative live birth, in all women except oocyte donors) were described. Statistical comparison between fertility diagnoses (i.e. female versus non-female infertility as described above in the population section) was performed using Wilcoxon and Mann-Whitney tests for continuous variables and Fisher's exact test for categorical variables. Bivariate correlation between POPs was assessed using Spearman's correlation analysis.

2.5. Single-pollutant models

Only POPs with detection rates above 75% were used in the statistical analysis with the aim of minimizing the impacts of left-censored data imputation on regression estimates (Schisterman et al., 2006). We evaluated the associations between each individual POPs and IVF outcomes using multivariate Poisson regression models with robust variance (Chen et al., 2018). Since we only used data from the first embryo transfer we did not account for the clustering effect of multiple cycles per women (Messerlian and Gaskins, 2017). Association estimates are summarized with beta (β) coefficients and relative risks (RR) with their respective 95% confidence intervals. Confounding variables considered in the first exploratory analysis included maternal age, BMI, smoking status and residential environment identified based on the most recently published literature (Lefebvre et al., 2021). Due to the amphiphilic nature of PFAS, the related models were also explored with serum lipids as confounders. On the basis of previous studies, we also analyzed the role of anti-Müllerian hormone (AMH) in the causal structure between POPs and IVF outcomes (Whitworth et al., 2015). No statistical association was found between POPs and AMH. However, some significant associations between AMH and some IVF outcomes were identified (Fig. S2). Thus, we ruled out the potential mediating role of AMH and considered it as potential confounder. In the final models we considered maternal age, BMI and AMH. We conducted sensitivity analyses, stratifying on 1) smoking status (only active smokers vs non and past smokers), 2) body mass index (BMI, <25 kg/m^2 vs >25 kg/m^2) and 3) infertility diagnoses (endometriosis, PCOS, unexplained infertility, premature ovarian insufficiency, combined infertility and non-female factor infertility [i.e. male infertility and egg donation]).

2.6. Multipollutant models

The chemical signatures between sub-groups of women on the basis of infertility sub-types (ie. male factor/donors, endometriosis, other female infertility) and their discriminant capacity were inspected using partial least squares discriminant analysis (PLSDA) with the R package "*ropls*". We conducted a multipollutant exploration with Bayesian Kernel Machine Regression (BKMR) (R package '*bkmr*') in order to assess the joint effect of chemicals and identify non-linear effects and interactions between chemicals. We considered two main outcomes for this analysis representative of intermediate outcomes (i.e. embryo quality) and clinical outcomes (i.e. live birth rate) using the Gaussian and *probit* (binomial) link function, respectively. We computed the methods in compound-wise variable selection mode considering default *prior* parameters and 10,000 iterations to ensure convergence. Posterior inclusion probabilities were used as a measure of relative importance of pollutants in the models. Four main groups of chemicals were established on the basis of structural similarities, namely a) dioxin-like PCBs, b) non dioxin-like PCBs, c) pesticides and d) PFAS. All statistical analyses were all performed using R version 4.0.3.

3. Results

3.1. Demographic characteristics, stimulation protocol and IVF outcomes

A total of 136 women were included in the present study. Demographic characteristics, ovarian stimulation protocol, intermediate and clinical IVF outcomes are presented in Table 1. The mean age of 136 included women was 33 (SD = 4) years and the mean BMI was 24 (SD = 5) kg/m^2. Approximately 60% of women never smoked and are current users of tampons. Women attended the IVF service to donate oocytes (11.8%), or had infertility caused by a male factor (10.3%), a female factor (37.5%), combined (25%) or unexplained infertility (15.4%). The mean mature oocyte retrieved and fertilization rate were 10 (SD = 5) and 0.7 (SD = 0.3), respectively. Live birth after the first embryo transfer was achieved by 32% of women (n = 37). There was no significant difference in the subgroup (n = 115) compared to our total population (n = 136).

Six PFAS out of the 14 analyzed congeners were frequently detected in serum samples (detection rates >75%) whereas the rest of them were found below the detection limits (Table 2). The compounds with highest concentrations were PFOS and PFOA, showing median values of 1.90 (1.42–2.52) and 1.08 (0.80–1.56) ng/g, respectively. Follicular concentration of 14 PCBs and 25 OCPs was available for 127 patients. The 14 PCBs were detected in all women. Seven OCPs out of 25 were detected in more than 75% of subjects, with lowest detection rates for gamma-hexachlorocyclohexane (γ-HCH) and trans-nonachlor (78% in both cases). The DDT metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (*p,p'*-DDE) showed the highest concentration levels among the pesticides (median 53.4 ng/g) followed by hexachlorobenzene (HCB, median 18.7 ng/g). Details of those observed distributions of internal exposure levels are presented in Table 2. Complete information on all POPs in serum and follicular fluid were available for 112 women. This subset of women with complete biomarker information did not differ from the whole study population in terms of POP levels. Strong correlation coefficients were found among chemicals from the major chemical families, especially among the groups: dioxin-like PCBs and trans-nonachlor, non-dioxin like PCBs (PCB28, 52, 101), pesticides and PFAS (Fig. 1).

Note: Per- and polyfluoroalkyl substances were measured in serum and organochlorinated chemicals in follicular fluid. Chemical abbreviations are defined in Table 2. Associations between serum levels of PFAs and IVF outcomes.

Associations between serum PFAS levels and IVF outcomes are summarized in Fig. 2A and B. Models adjusted for maternal BMI, age and AMH resulted in non-statistically significant associations between PFAS and most intermediate (Fig. 2A) and all clinical IVF outcomes (Fig. 2B). Only PFNA and PFDA showed positive associations with the number of useable blastocysts, (β 0.26, 95%CI [0.03; 0.49], $p = 0.027$, and β 0.21, 95%CI [0.00; 0.42], $p = 0.047$ respectively). The results from regression models without AMH are displayed in Fig. S3, showing some additional

Table 1
Demographic and reproductive characteristics of women attending *in vitro* fertilization (IVF) included in the study (n = 136).

Demographic and lifestyle characteristics	N	Mean (standard deviation) or n (%)
Maternal Age (years)	136	34 (4)
Maternal BMI (kg/cm^2)	136	24 (5)
Weight gain >5 kg	130	53 (39%)
Weight loss >5 kg	130	16 (12%)
Maternal smoking status	136	
Never		81 (59.6%) [a]
Past		35 (25.7%) [a]
Active		20 (14.7%) [a]
Education Level	136	
Primary and secondary		35 (25.7%) [a]
University		101 (74.3%) [a]
Current living environment	136	
Rural		52 (38.2%) [a]
Semi-urban		15 (11.1%) [a]
Urban		69 (50.7%) [a]
Physical Activity	133	
None		7 (5.3%) [a]
High		82 (61.7%) [a]
Moderate		44 (33%) [a]
Use of tampons	135	
No		49 (36.3%) [a]
Past		8 (5.9%) [a]
Yes		78 (57.8%) [a]
Past Use of oral contraceptive pill		
Yes	135	130 (96.3%) [a]
Duration (years)	123	9 (5) [a]
Breastfeeding	134	23 (17.2%)
Etiological characteristics		
Etiology	136	
Oocyte donor		16 (11.8%) [a]
Male factor		14 (10.3%) [a]
Female factor		
Tubal factor		2 (1.5%)
Endometriosis		19 (14%)
Ovulatory factor		30 (22%)
Combined infertility		34 (25%) [a]
Unexplained		21 (15.4%) [a]
Duration of infertility (months)	136	47 (30) [a]
Type of infertility	136	
Primary		67 (49.3%) [a]
Primary-secondary		24 (17.6%) [a]
Secondary		45 (33.1%) [a]
Anti-Müllerian hormone (AMH, ng/ml)	136	2.8 (2.4)
Antral Follicle Count (AFC, n)	134	22.4 (12.5)
Ovarian Stimulation protocol		
IVF cycle rank	136	1.6 (0.9)
Ovarian stimulation protocol	136	
Agonist		7 (5%) [a]
Antagonist		129 (95%) [a]
Duration of stimulation (days)	136	11.0 (1.4)
Total dose of gonadotrophins	136	2500 (726)
Intermediate IVF outcomes		
Total Number of oocytes retrieved	136	12 (6)
Number of mature oocyte retrieved	136	10 (5)
Oocyte maturity rate	136	0.8 (0.2)
Fertilization rate	136	0.7 (0.3)
Number of utile embryo	136	2.9 (2.6)
Day 5 blastocyste useable rate (D5BUR)	136	0.3 (0.25)
Clinical IVF outcomes – 1st embryo transfer		
Positive pregnancy test	115	44 (38.3%)
Implantation rate	115	41 (35.7%)
Clinical pregnancy	115	39 (33.9%)
Miscarriages	115	8 (7%)
Live birth 1st transfer	115	37 (32.2%)
Clinical IVF outcomes – all embryo transfers		
Cumulative Live birth rate	115	50 (43.5%)

Abbreviations: BMI body mass Index; IVF *in vitro* fertilization.
Internal exposure levels of POPs in blood and follicular fluid.
[a] Calculated in all patients except egg donors.

Table 2
Distributions of persistent organic pollutants (POPs) detected above 75% of samples in serum and follicular fluid of women attending *in vitro* fertilization.

Chemical	<LOD n (%)	Units	Median	Interquartile Range
Per- and polyfluoroalkyl substances (n = 136) Serum				
PFOS	0	ng/g	1.90	1.42–2.52
PFOA	0	ng/g	1.08	0.80–1.56
PFHxS	0	ng/g	0.78	0.58–1.01
PFNA	0	ng/g	0.41	0.32–0.58
PFDA	1 (0.7%)	ng/g	0.20	0.14–0.25
PFUnA	12 (8.8%)	ng/g	0.16	0.12–0.22
Organochlorinated chemicals (n = 127) Follicular Fluid				
PCB105	0	pg/g	1.12	0.83–1.69
PCB114	0	pg/g	0.20	0.14–0.29
PCB118	0	pg/g	6.52	4.56–9.14
PCB123	0	pg/g	0.10	0.06–0.13
PCB156	0	pg/g	2.08	1.32–2.89
PCB157	0	pg/g	0.43	0.30–0.67
PCB167	0	pg/g	1.09	0.76–1.56
PCB189	0	pg/g	0.24	0.16–0.37
∑Non-Coplanar PCBs	0	pg/g	12.10	8.20–15.68
PCB28	0	pg/g	1.02	0.73–1.50
PCB52	0	pg/g	0.42	0.31–0.60
PCB101	0	pg/g	0.55	0.41–0.77
PCB138	0	pg/g	16.00	11.48–20.71
PCB153	0	pg/g	26.30	18.00–35.76
PCB180	0	pg/g	18.00	12.01–27.83
∑Non-dioxin like PCBs	0	pg/g	64.30	43.30–85.31
PeCBz	4 (3.1%)	pg/g	0.51	0.36–0.83
HCB	0	pg/g	18.7	15.18–22.86
g-HCH	28 (22.0%)	pg/g	1.47	0.64–2.89
β-HCH	0	pg/g	6.90	4.86–10.48
Epoxyheptachlor	1 (0.8%)	pg/g	2.77	2.17–3.72
Trans-nonachlor	28 (22.0%)	pg/g	2.00	1.41–2.55
p,p'-DDE	0	pg/g	53.4	38.82–84.61

Abbreviations: b-HCH, beta-hexachlorocyclohexane; HCB, hexachlorobenzene; PCB, polychlorinated biphenyl; PeCBz, pentachlorobenzene; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFUnA, perfluoro-n-undecanoic acid; *p,p'*-DDE, p,p'dichlorodiphenyldichloroethylene.
Associations are represented by their beta coefficients (β) or relative risks (RR) with 95% confidence intervals (CI) from models adjusted for age, body mass index and the levels of anti-Müllerian hormone. Intermediary outcomes (Panel A) included number of oocyte retrieved (ORT), oocyte maturity rate (MR), fertilization rate (FR), number of useable blastocyst (UB) and day 5 useable blastocyst rate (D5BUR) and clinical outcomes (Panel B), implantation rate (IR), pregnancy rate (PR), clinical pregnancy rate (CP), live birth rate after 1st blastocyst transfer (LB), cumulative live births (LBc). Statistically significant ($p < 0.05$) associations are depicted with white circles. Abbreviations of chemicals are detailed in Table 2 with white circles. Abbreviations of chemicals are detailed in Table 2.

PFAS positively associated with ORT and useable blastocysts. Stratification analysis for BMI, smoking and infertility showed similar trends (data not shown).

Associations between follicular fluid concentrations of organochlorinated chemicals and IVF outcomes.

The associations between follicular levels of PCBs and OCP with IVF outcomes are summarized in Fig. 3A and B, for intermediate and clinical outcomes respectively. No significant associations were found between organochlorinated chemicals and oocyte maturity rate, fertilization rate or D5BUR. Follicular fluid levels of PCB 189 and PCB 52 were significantly associated with the number of oocyte retrieved (β 0.10, 95%CI [0.00; 0.19], $p = 0.04$ and β 0.09, 95%CI [0.00; 0.17], $p = 0.038$ respectively). Conversely, PCB52 was also positively associated with the number of useable blastocysts (β 0.24, 95%CI [0.08; 0.40], p = 0.004). Conversely, negative associations between PCB138 and trans-nonachlor with useable blastocysts were identified, β −0.28 (95%CI [−0.52; −0.04] $p = 0.02$) and β −0.22 (95%CI [−0.40; −0.03] $p = 0.02$), respectively. Adjustment for AMH had relevant impact on the

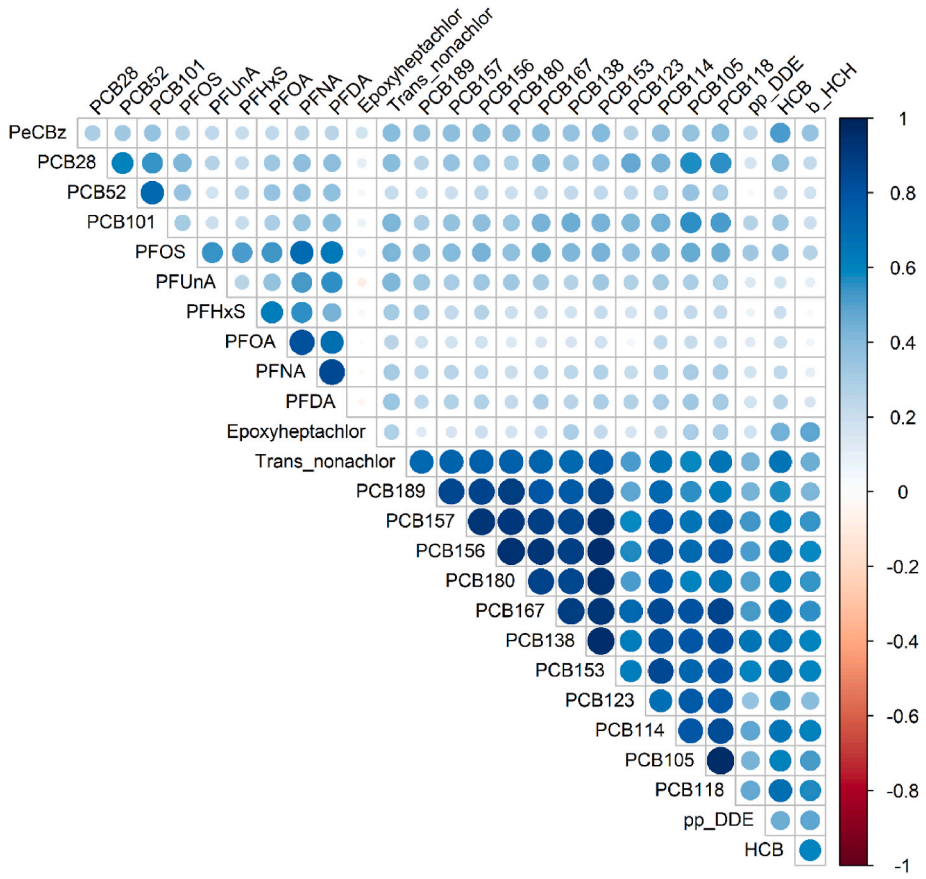


Fig. 1. Heatmap representing the bivariate correlation between persistent organic pollutants.

associations between POPs and the number of oocyte retrieved and useable blastocysts. For instance, the models not adjusted for AMH tended to reveal positive associations for POPs with the number of oocyte retrieved or nullified some negative associations between PCBs (118, 167 and 138) with the number of useable blastocysts (Fig. S4). No significant associations were found between clinical IVF outcomes and follicular levels of most organochlorinated chemicals (Fig. 3B), with the exception of PCB28 that showed an increase of clinical pregnancies (RR 1.49 [1.09; 2.03], $p = 0.01$), live birth rates (either at first attempt RR 1.63 [1.24; 2.16], $p = 0.001$ and cumulative live birth rates: RR 1.42 [1.11; 1.83], $p = 0.005$). Stratification by BMI, smoking and infertility diagnoses did not show evidence of effect modification (data not shown).

In order to explore the joint effect of POPs, non-linearity and interactions between chemicals, we conducted a multipollutant analysis using BKMR within a subset of 105 individuals having complete information on both group of biomarkers (PFAS and organochlorinated chemicals) and the outcomes considered for this analysis, number of useable blastocysts and live birth rates. The model for live birth was conducted with hierarchical variable selection, being PCB28 the chemical with largest posterior inclusion probabilities, above 0.5 (Fig. 4A). The univariate exposure-response functions for the top 6 chemicals are shown in Fig. 4B, which can be interpreted as the relationship between chemicals and live birth rates in IVF when all other chemicals are held at their median levels. Consistent with the single pollutant analysis, PCB 28 was associated a reduction of live births. The cumulative effect of the overall chemical mixture on live birth is depicted in Fig. 4C, showing a slightly upward non-significant curve. Finally, individual exposure-response functions for the top 6 contributing chemicals are depicted in Fig. 4D across percentile 25, 50 and 75 of the other chemicals, when the rest are fixed to the median. This inspection did not elucidate suspected interactions between chemicals nor non-monotonic relationships in specific cross-sections. Additional analysis with intermediate outcomes (i.e. number of useable blastocysts) and on pregnancy rates resulted in similar association patterns (data not shown). The results from PLSDA showed that chemical signatures poorly discriminated female subgroups supporting the fact that chemical mixtures were homogeneous between females with different underlying conditions (Fig. S5).

4. Discussion

To our knowledge, the present study is the first to demonstrate that French women undergoing IVF are exposed to mixtures of POPs, including historical organochlorinated chemicals and more emerging compounds like PFAS. Specifically, we provide here original data on the levels of trans-nonachlor or epoxy-heptachlor and their associations with IVF outcomes. Multivariate regression models showed inconsistent association pattern across congeners and IVF outcomes. The most consistent findings across outcomes was found for PCB28 that showed positive associations with the number of useable blastocysts, pregnancy rate and live birth rate. The number of useable blastocysts was also negatively associated with PCB118, PCB167, PCB138 and trans-nonachlor levels. The results from the BKMR analysis supported the lack of association of the mixture with intermediate and clinical outcomes.

4.1. Internal exposure levels of POPs in blood and follicular fluid

The PFAS we analyzed and levels found in the present study are in the range of those found in the same period among the general French population (Fillol et al., 2021) and women with and without endometriosis from the same region (Matta et al., 2022). The congeners of PFAs

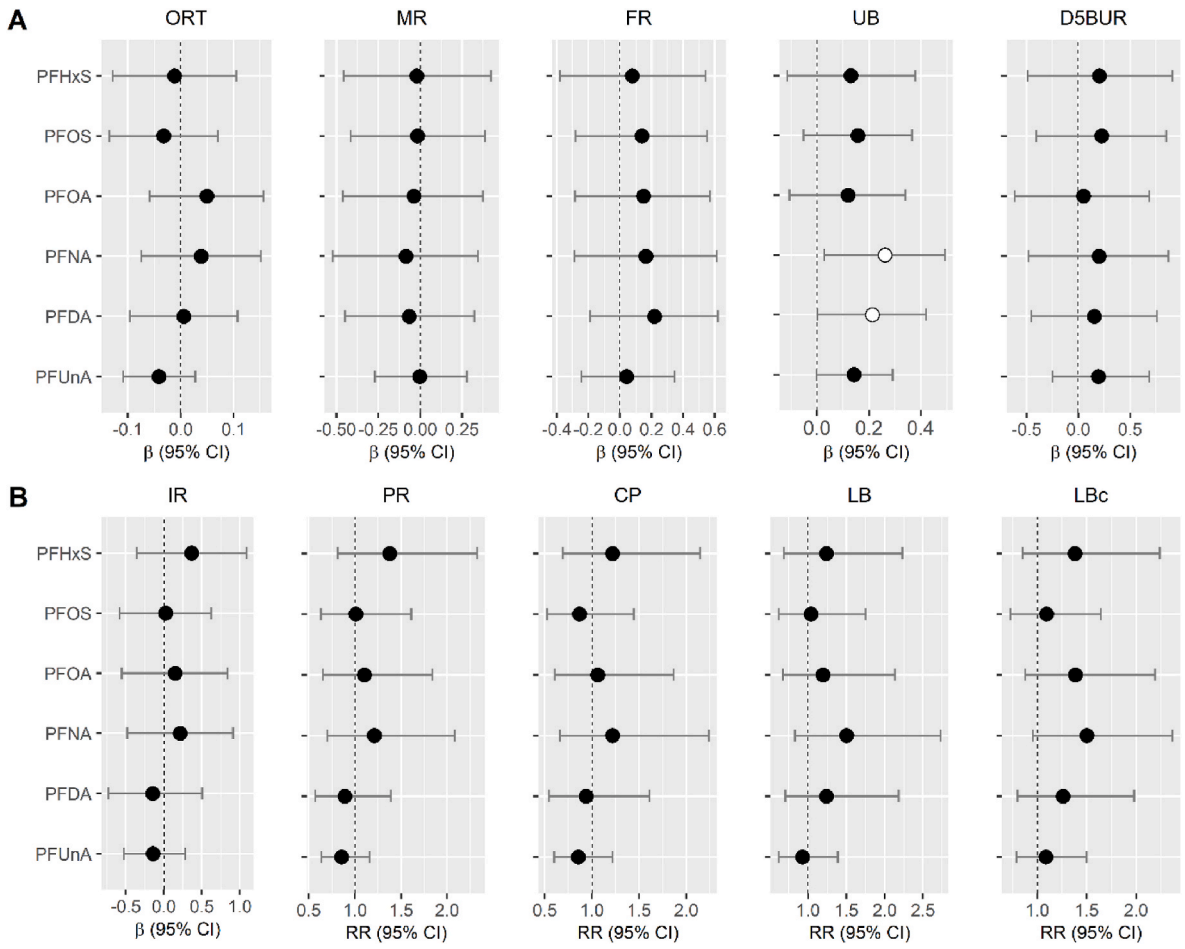


Fig. 2. Forest plots depicting the associations between per- and polyfluoroalkyl substances in serum with *in vitro* fertilization (IVF) outcomes.

found above the detection limit were mostly the same in previous studies performed on this topic in other countries, although levels were often 1–10 fold lower (Governini et al., 2011; McCoy et al., 2017; Kim et al., 2020; Petro et al., 2014; Wang et al., 2021). The concentrations of PCBs and OCPs in follicular fluid found in the present study are lower than previous studies assessing IVF outcomes (Petro et al., 2012; Weiss et al., 2006; Younglai et al., 2002). We also detected heterogeneity among previous studies in the timing of the blood sampling during ovarian stimulation (Lefebvre et al., 2021). Although some studies reported stability of serum POP levels throughout ovarian stimulation (Petro et al., 2012; McCoy et al., 2017), it might be speculated that the levels of lipophilic POPs might slightly vary via a mobilization of lipids induced by the hormonal status. In this study we carried out blood sampling on the first day of stimulation, in the same conditions and with the same assay for all the women.

4.2. Associations between internal levels of PFAs and IVF outcomes

Previous studies evaluated the association between follicular levels of PFAS and IVF outcomes (Governini et al., 2011; Kim et al., 2020; McCoy et al., 2017; Petro et al., 2014; Björvang et al., 2021). One of them was also conducted in serum (McCoy et al., 2017). The levels of PFAS have mostly been reported to be very similar between matrices, although some congeners (i.e. PFUnA) was found at lower concentrations in follicular fluid than in plasma (McCoy et al., 2017). Overall, we did not find statistically significant associations between PFAS levels and intermediary or clinical IVF outcomes, consistently with the existing literature, as synthesized in our recent systematic review (Lefebvre et al., 2021). Concerning intermediate IVF outcomes, we found statistically significant positive associations between PFNA and PDFA levels and UB. Although endpoints are not strictly comparable, this might be considered as consistent with another previous study reporting an association between overall PFAS levels and number of good quality embryos (Petro et al., 2014). However, two recent studies reported conflicting results, as PFAs levels were associated with a lower number of good quality embryo (Ma et al., 2021) and lower average embryo score (Björvang et al., 2021). It is difficult to raise hypotheses on the reasons explaining these heterogeneous findings, but the different endpoints and embryonic stages evaluated across these studies could potentially account for these discrepancies. Noteworthy, our study was the only one to focus on blastocysts, and to use D5BUR as an endpoint, which is a relevant and validated tool (Hammond, 2019).

4.3. Associations between internal concentrations of organochlorinated chemicals and IVF outcomes

Previous studies were consistent our findings, globally reporting null associations between PCBs and OCPs (mostly p,p'-DDE) with IVF outcomes (Lefebvre et al., 2021). For instance, only two out of six previous studies on OCPs have reported that fertilization rate was negatively associated with p,p'-DDE or the principal component representing the pesticides (Petro et al., 2012; Younglai et al., 2002). Concerning embryo quality, only 2 studies out of 7 reported a negative association with OCP (Petro et al., 2012; Björvang et al., 2021) and 3 out of 5 reported significant negative associations with PCBs (Bloom et al., 2017; Petro et al., 2012; Björvang et al., 2021); the other reported no association (Lefebvre et al., 2021). Concerning clinical IVF outcomes, results in the literature are also discordant, but most of them have reported null associations.

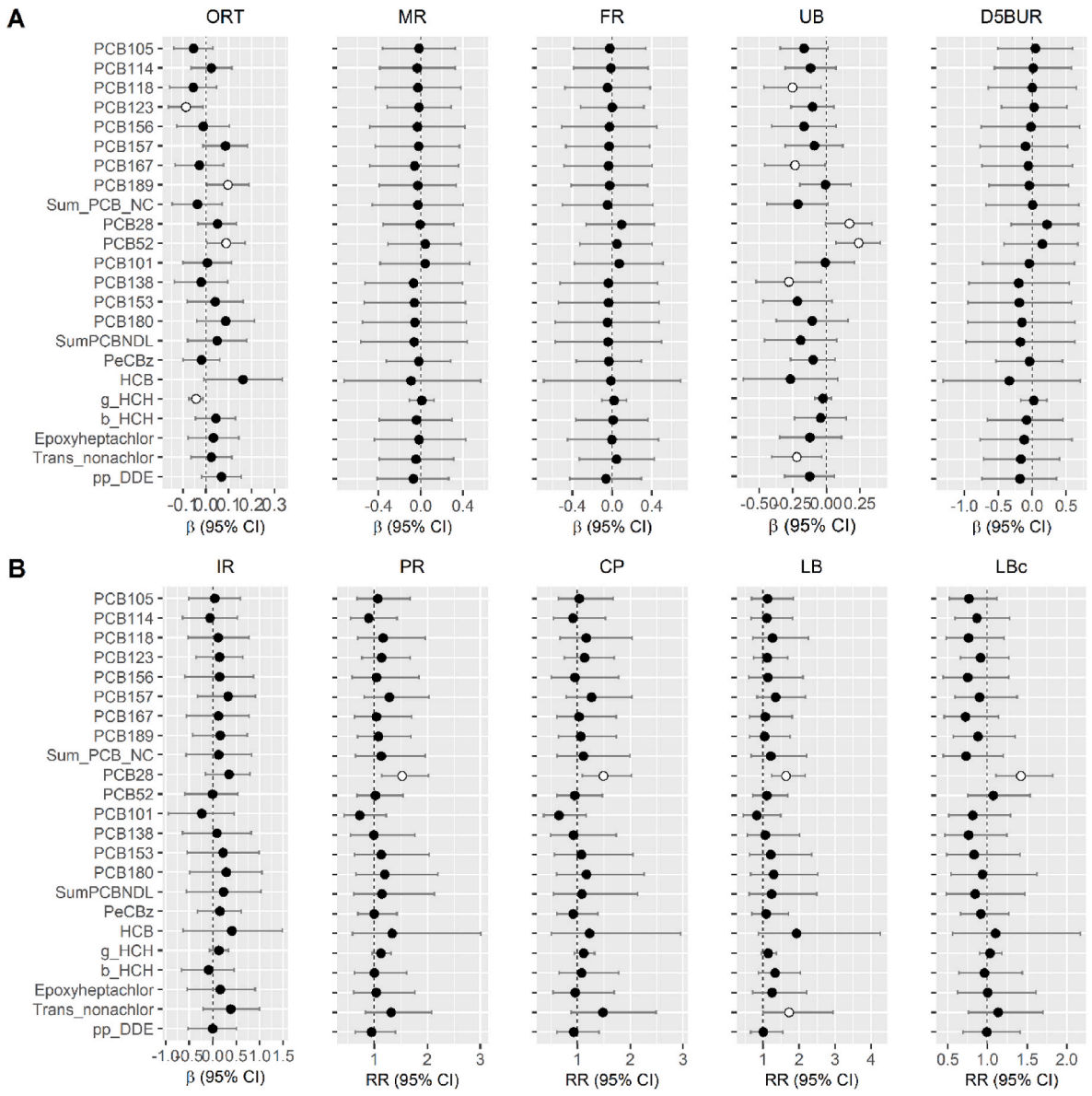


Fig. 3. Forest plots depicting the associations between organochlorinated chemicals in follicular fluid with *in vitro* fertilization (IVF) outcomes. Associations are represented by their beta coefficients (β) and relative risks (RR) with 95% confidence intervals (CI) from models adjusted for age, body mass index and the levels of anti-Müllerian hormone. Intermediary outcomes (Panel A) included number of oocyte retrieved (ORT), oocyte maturity rate (MR), fertilization rate (FR), number of useable blastocyst (UB) and day 5 useable blastocyst rate (D5BUR) and clinical outcomes (Panel B), implantation rate (IR), pregnancy rate (PR), clinical pregnancy rate (CP), live birth rate after 1st blastocyst transfer (LB), cumulative live births (LBc). Statistically significant ($p < 0.05$) associations are depicted with white circles. Abbreviations of chemicals are detailed in Table 2.

The most statistically powered studies have shown an increase of the odds of failed implantation related with the serum concentrations of PCB153 or the sum of PCBs (Meeker et al., 2011) and the pesticide HCB (Mahalingaiah et al., 2012). The IVF outcome acknowledged to be the most clinically relevant is live birth rate, but it has been measured in a minor extend in previous studies (5 on PCBs and 3 on OCPs). Among these studies three have reported negative associations between one or more PCBs with live birth rates or odds (Meeker et al., 2011; Bloom et al., 2017; Björvang et al., 2021) and one study found positive associations with *p,p'*-DDE (Bloom et al., 2017), whereas the rest showed null results.

Some sources of heterogeneity have been previously identified to help explaining the inconsistencies across studies (Lefebvre et al., 2021). For instance, the different geographical areas determine different dietary habits, lifestyles and ethnicities, that may impact the pattern of co-exposures and presence of other underlying factors associated with IVF outcomes (Humphries et al., 2016; James-Todd et al., 2016). The difference between the populations in terms of infertility etiology, maternal BMI, age or smoking status may also contribute to this heterogeneity (Ribeiro et al., 2022; Luke et al., 2012). Female included in our study were quite homogenous in terms of origin and residence area or age range. In addition, we may also highlight the different congeners analyzed or detected, especially in case of PCBs, that challenge the direct comparison between studies.

4.4. Limitations and strengths

We acknowledge some limitations for the present work. First of all, this is an exploratory study with a relatively limited sample size, especially to identify modest effects or explore associations across subgroups. For instance, the limited sample size prevented us from conducting interaction analysis between chemical exposures and dietary

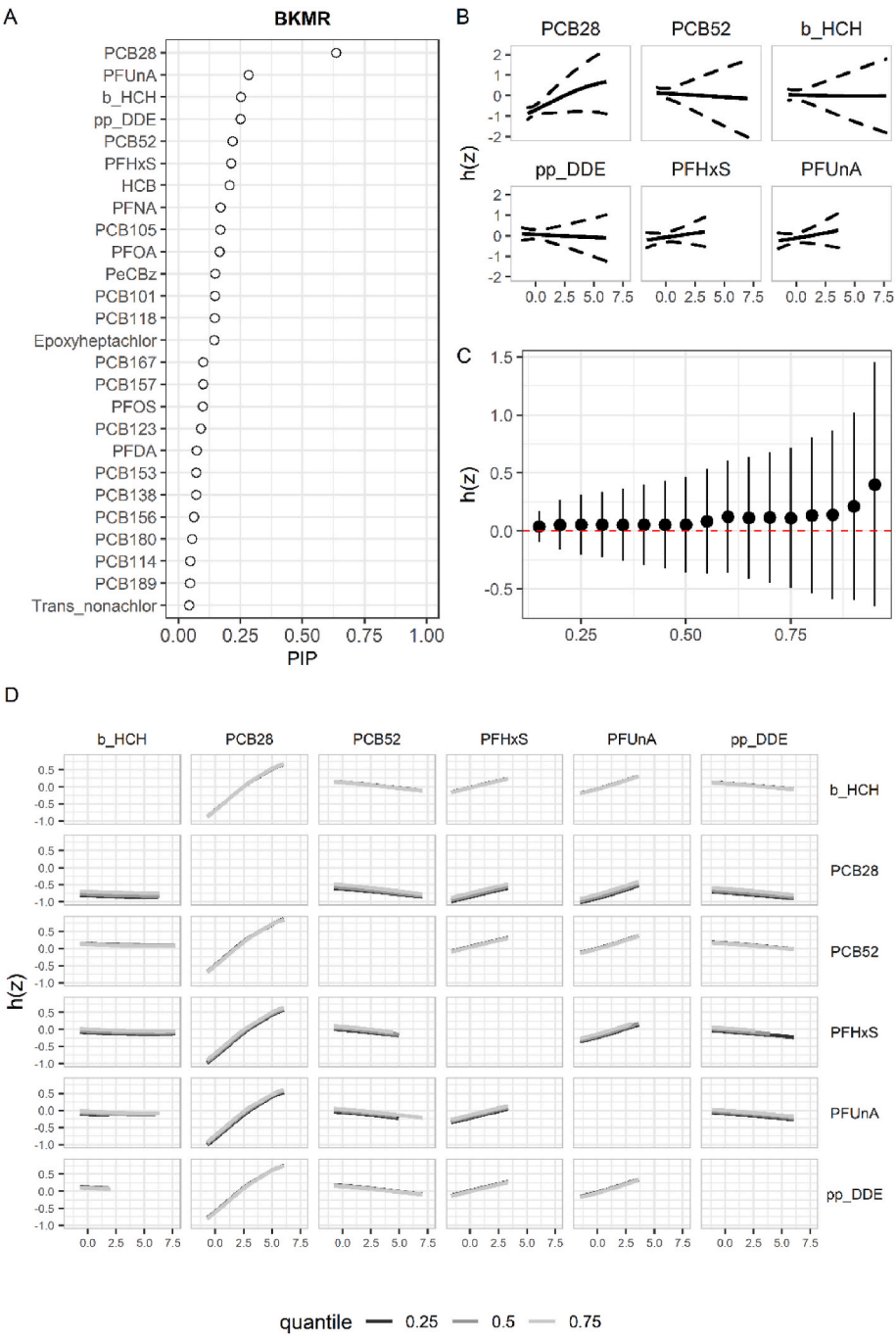


Fig. 4. Summary estimates from Bayesian Kernel Machine Regression (BKMR) analysis on the associations between persistent organic pollutants and live birth. The ranking of variable contributions to the model is depicted by Posterior inclusion probabilities (PIP) in panel A. Single exposure estimates for most relevant variables are detailed in Panel B. The joint effect of the mixture is shown in Panel C. Cross-sections of individual exposure-response functions across quantiles of other exposure is detailed in panel D. For interpretation, unparalleled and crossing lines may suggest the presence of interactions between chemical pairs. Abbreviations of chemicals are detailed in Table 2.

habits that could be either protective or harmful, thus potentially modifying the overall effect estimates. We also included all women undergoing IVF, including infertile women with various etiologies, but also women without known female infertility factors such as oocyte donors, which may contribute to the heterogeneity of our findings. In order to overcome this limitation, we performed sensitivity analyses excluding women subgroups on the basis of infertility subtypes without major impacts on the overall findings. In addition, we chose to analyze only women who had undergone stimulation with oocyte retrieval. We therefore have no information on the potential associations between POPs exposure levels and premature termination of treatment for non-response to stimulation in particular, thus selection bias cannot be ruled out (Messerlian and Gaskins, 2017). The concern about the impact about the male factor on the IVF outcomes has been growing, but to date the evidence seems limited as the majority of male infertility cases are idiopathic (Mazzilli et al., 2017). In our study, the number of IVF cycles with present male infertility was too limited, nonetheless, further studies with suitable recruitment settings should strongly consider it. Finally, we should also acknowledge the complexity of modelling the IVF process when multiple cycles per women are involved, resulting in measurement errors related to the 'unit of analysis' (Missmer et al., 2011). Despite the fact there is no consensus on the best approach and the present study involved women with only one cycle, we opted to use two metrics (p.e. First transfer and cumulative live birth) that showed similar results.

We should also highlight some strengths of this study. We have conducted a comprehensive characterization of POPs in serum and follicular fluid of women attending IVF, including pesticides never

explored before (trans-nonachlor and epoxy-heptachlor). To this end, we have applied highly sensitive ultra-trace methods allowing the characterization of a large number of congeners at very low concentrations. Additionally, we have applied a robust multipollutant analysis using BKMR, a powerful method to identify joint effects, interactions and non-linear effects, overcoming the high collinearity related to exposure data (Bobb et al., 2015). We should also highlight the large panel of intermediary and clinical outcomes covering the entire IVF process, conducted with standardized protocols and harmonized international classifications, including D5BUR, a novel validated performance indicator of adverse embryology outcomes (Hammond, 2019).

5. Conclusion

To conclude, the present study represents the first one conducted in France characterizing the internal exposure of women to POPs at reproductive age and its associations with IVF outcomes. The results showed that mixtures of low concentrations of PCBs, OCPs and PFAS are widespread among women. Most POPs were not associated with the most clinically relevant IVF outcomes, like live birth rates. However a number of the measured PCBs and the pesticide trans-nonachlor were negatively associated with the number of useable blastocyst. Due to the high societal and economical cost associated to infertility and failed IVF further research is urged. Future studies should be conducted in a large population sample in order to ensure the statistical power to identify modest effects and a robust stratification analysis to characterize the sources of heterogeneity.

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Author statement

TL: Conceptualization; Data curation; Formal analysis; Roles/Writing - original draft. TF, Resources; Supervision; Validation; Writing - review & editing. GD: Data curation; Investigation; SP: Resources; Supervision; Validation; Writing - review & editing. PM: Formal analysis; Investigation; Methodology. BLB: Resources; Supervision; Validation; Writing - review & editing. JPA: Conceptualization; Resources; Supervision; Validation; Writing - review & editing. GCS: Conceptualization; Funding acquisition; Validation; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120087.

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Associations between persistent organic pollutants and endometriosis: A multiblock approach integrating metabolic and cytokine profiling

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ABSTRACT

Humans are exposed daily to complex mixtures of chemical pollutants through their environment and diet, some of which have the potential to disrupt the bodies' natural endocrine functions and contribute to reproductive diseases like endometriosis. Increasing epidemiological and experimental evidence supports the association between endometriosis and certain persistent organic pollutants (POPs) like dioxins; however, little is known about the underlying linking mechanisms. The main objective of this study is to proof the methodological applicability and discovery potential of integrating ultra-trace mass spectrometry (MS) profiling of POP biomarkers and endogenous biomarker profiling (MS metabolomics and cytokines) in a case-control study for the etiological research of endometriosis. The approach is applied in a pilot clinical-based study conducted in France where women with and without surgically confirmed endometriosis were recruited. Serum samples were analysed with high-resolution MS for about 30 polychlorinated biphenyls (PCBs), organochlorinated pesticides and perfluoroalkyl substances (PFAS). About 600 serum metabolites and lipids were identified with targeted metabolomics using tandem MS with the Biocrates MxP® Quant 500 Kit. A panel of 4 pro-inflammatory cytokines were analysed using ELISA-based 4-PLEX analyser. Statistical analysis included a battery of variable selection approaches, multivariate logistic regression for single-chemical associations, Bayesian kernel machine regressions (BKMR) to identify mixture effects of POPs and a multiblock approach to identify shared biomarker signatures among high risk clusters. The results showed the positive associations between some POPs and endometriosis risk, including the pesticide *trans*-nonachlor Odds Ratio (95% Confidence Interval) 3.38 (2.06–5.98), $p < 0.0001$ and PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$. The BKMR approach showed a tendency of a positive cumulative effect of the mixture, however *trans*-nonachlor exhibited significant associations within the mixture and interacted with other PCBs, strengthening the effects at highest concentrations. Finally, the multiblock analysis, relating the various blocks of data, revealed a latent cluster of women with higher risk of endometrioma presenting higher concentrations of *trans*-nonachlor, PCB 114 and dioxin-like toxic equivalents from PCBs, together with an increased inflammatory profile (i.e. elevated interleukin-8 and monocyte chemoattractant protein-1). It was also highlighted a specific metabolic pattern characterized by dysregulation of bile acid homeostasis and lipase activity. Further research will be required with larger sample size to confirm these findings and gain insight on the underlying mechanisms between POPs and endometriosis.

1. Introduction

Humans today are daily exposed to a plethora of environmental contaminants, some of which have the potential to disrupt our bodies' natural hormonal regulations and pose a threat to human health (Diamanti-Kandarakis et al. 2009; Kahn et al. 2020). A group of endocrine

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disrupting chemicals (EDCs) known as Persistent Organic Pollutants (POPs) is of particular concern due to their bioaccumulative properties and impacts on human health (UNEP 2017). Women have been shown to be especially vulnerable to the effects of EDCs which have been related to a handful of female reproductive systems including hormone-sensitive cancers, infertility, disruption of menstrual cycles or endometriosis (Gore et al. 2015; Green et al. 2021; Kahn et al. 2020).

Epidemiologic and experimental evidence exists linking exposure to POPs with endometriosis (Cano-Sancho et al. 2019; Matta et al. 2021), an oestrogen regulated disease characterised by the presence of endometrial tissues outside of the uterus, and highly variable and non-specific symptoms like chronic pelvis pain, dysmenorrhea or dyschesia (Giudice 2010). Despite it is commonly considered a benign pathology, endometriosis is associated with higher risk of ovarian and breast cancers, asthma, and higher risk of several chronic diseases such as asthma and cardiovascular diseases (Kvaskoff et al. 2015; Kvaskoff et al. 2021). Endometriosis is estimated to impact 5–15% of women who menstruate (Shafrir et al. 2018).

The highly active 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is by far the most studied pollutant in association with endometriosis (Matta et al. 2021), with limited studies on the rest of POPs individually or as mixtures. Additionally, the mechanisms of action of POPs remains largely unknown partially due to the constraints of animal models to fully recapitulate the pathogenesis undergoing in women (Grümmer 2006). Over the past decades, new high throughput technologies have emerged, allowing us to study endometriosis with more molecular scrutiny, including metabolic profiling (e.g. metabolomics). The identification of predictive "omic" biomarkers of endometriosis has been an active field of research, motivated by the long and expensive diagnosis delays, lasting between 8 and 10 years. Some recent studies using metabolomic profiling have revealed an altered lipid profile associated to the development and progression of endometriosis (Cordeiro et al. 2015; Domínguez et al. 2017). In turn, metabolomics is also gaining popularity in the field of environmental health research as a cost-efficient means for linking external with internal exposures, biological responses and diseases (Vermeulen et al. 2020; Walker et al. 2019), proved as promising by few applied studies (Jin et al. 2020; Stratakis et al. 2020).

To our knowledge, no study has yet attempted to integrate biomarkers of exposure to POPs with metabolic and cytokine profiling to uncover signatures potentially informing about molecular pathways underlying the progression of endometriosis. Thus the main objective of this pilot study is to proof the methodological applicability and discovery potential of integrating ultra-trace profiling of POP biomarkers and endogenous biomarker profiling (metabolomics and cytokines) in the etiological research of endometriosis.

2. Methods and materials

2.1. Study design and recruitment

The ENDOXOMICS-β study is an ongoing clinical-based case-control study conducted at the Gynecology-Obstetrics Department and Biology and Reproductive Medicine Departments from the Univesity Hospital of Nantes, France. For the present study, 87 participants were recruited during 2018–2019 including only adult female patients (between 18 and 45 years) seeking surgical intervention on deep endometriosis without endometrioma (noOMA), with endometrioma (OMA) or other benign reproductive issues not related to endometriosis (controls). Endometriosis diagnoses for cases were performed by laparoscopy with histological confirmation Gynecology-Obstetrics Department as described elsewhere (Ploteau et al. 2017). All cases presented severe forms of endometriosis classified in stages III or IV according to the American Fertility Society reviewed-classification (AFSr) scale and often they were cases with rectosigmoid deep endometriosis (DE) exhibiting frozen pelvis with abundant adhesions. Controls comprised similar aged women consulting for non-endometriosis related benign gynaecological issues exhibiting no endometriosis-like symptoms, including tubal ligation or fertile women undergoing assisted reproductive technology for male infertility or oocyte donation. Fibroids or polycystic ovarian syndrome (PCOs) were not eligible. All patients gave informed consent to participate in the study; patients who did not provide written consent were excluded. The protocol was approved by the local biotethics committee Groupe Nantais d'éthique dans le Domaine de la Santé (GNEDS). Each participant provided a serum sample (at least 5 mL) that was stored at −80 °C until the analysis in aliquots to minimize the freeze–thaw cycling. Each participant was interviewed by the clinicians during consultation using a questionnaire was adapted from the World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonization Project (EPHect) Questionnaire (Becker et al. 2014; Fassbender et al. 2014; Rahmioglu et al. 2014; Vitonis et al. 2014) about individual, socio-economic and demographic variables including age (years), body mass index (BMI, Kg/m^2), breast-feeding, parity, contraceptive use or type of infertility, among others.

2.2. Laboratory analysis

2.2.1. Characterization of exposure biomarkers of POPs

Concentrations of 20 polychlorinated biphenyls (PCBs), and 30 organochlorine pesticides (OCPs) were analysed in thawed serum samples using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) as detailed elsewhere (Koual et al. 2019; Ploteau et al. 2017). Serum concentrations of 14 perfluoroalkyl substances (PFAS) were quantified using liquid chromatography with tandem-mass spectrometry (LC-MS/MS) as previously detailed (Mancini et al. 2020). Briefly, serum samples underwent a preliminary alkaline digestion followed by a two-stage Solid Phase Extraction purification using polymeric Oasis® HLB and graphitised carbon (ENVI-Carb®) cartridges, and were subsequently analysed by LC-ESI(-)-MS/MS for PFAS. For dioxin-like (DL) and non-dioxin-like (NDL) PCBs, extracts were analysed by gas chromatography (7890A; Agilent Technologies, USA) coupled to a high-resolution mass spectrometer, double sector (JMS-700D and 800D; Jeol, Japan) operating at a resolution of 10,000 (10% valley) (Rivière et al. 2014). Pesticides were separated by capillary gas chromatography (Trace GC Ultra Thermo Scientific) equipped with a programmed temperature vaporiser (PTV) injector and coupled to a Quantum XLS Triple Quadrupole (GC–MS/MS). The mass spectrometer was operated in electron ionization (EI) mode. All analyses were performed with blinding under ISO17025:2005 standard conditions.

Concentration of organochlorinated POPs (PCBs and OCPs) were normalised based on total serum lipids, due to the bioaccumulative properties. Serum lipid content was determined with enzymatic kits (Biolabo, Maizy, France) as the sum of phospholipids, triglycerides, total cholesterol and free cholesterol (Akins et al. 1989). Concentrations below the limit of detection (LOD) were assigned a value of LOD/2 (medium bound approach).

2.2.2. Characterization of endogenous biomarkers

2.2.2.1. Targeted metabolomics profiling. Targeted metabolomics profiling was performed using the MxP® Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) in thawed serum aliquots. The kit allows the identification and quantification up to 630 endogenous metabolites and small molecules from 26 biochemical classes, combining the high throughput capacities of non-targeted methods with the absolute quantification capacities of classical targeted methods. Among the metabolites includes 14 classes of small molecules: alkaloids (1), amine oxides (1), amino acids (20), amino acid related (30), bile acids (14), biogenic amines (9), carbohydrates and related (1), carboxylic acids (7), cresols (1), fatty acids (12), hormones and related (4), indoles and derivatives (4), nucleobases and related (2), and vitamins and cofactors

(1); and 12 classes of lipids: acylcarnitines (40), lysophosphatidylcholines (14), phosphatidylcholines (76), sphingomyelins (15), ceramides (28), dihydroceramides (8), hexosylceramides (19), dihexosylceramides (9), trihexosylceramides (6), cholesteryl esters (22), diglycerides (44), and triglycerides (242). Small molecules were measured by LC-MS/MS and lipids were measured by flow injection analysis (FIA)-MS/MS using a 6500 + QTRAP® instrument (AB Sciex, Darmstadt, Germany) with an electrospray ionization source, using the 96-well plate kit. The targeted metabolomics analysis methods using the Biocrates Kit followed the extensively documented protocol in the user manual and is described below. For the purposes of the present study only FIA metabolites were used and related methodology is described.

FIA Solvent constituted the Biocrates FIA Mobile Phase Additive and 290 mL methanol (MeOH). The column was prewashed with 95% Solution B for 20 min with a flow rate of 0.5 mL/min. For the system suitability test (SST) and instrument calibration, the column was equilibrated with starting condition: 100% Solvent A with a flow rate of 800 μL/min at 50° C. SST for FIA consisted of injection of: 3 blanks, 3 test mixes, followed by 2 blanks, with 20 μL injection volume. Quality controls (QCs) were dissolved in 100 μL HPLC grade water. Internal standard (ISTD) mix was dissolved in 1200 μL HPLC water. All vials (Cal, QCs, and ISTD) were shaken for 15 min at 1200 rpm and vortexed. QCs and serum samples were centrifuged for 5 min at 2750 × *g* at 4° C. 10 μL of the ISTD mix was added to each well on the kit (except in the first double blank well) using an Eppendorf Multipette® E3 at maximum dispensing speed. 10 μL of the phosphate buffered saline solution, Cal, and QC were then pipetted according to the well plate layout. All wells were dried under nitrogen for 30 min using a nitrogen evaporator.

The derivatisation solution was prepared using 1900 μL of each: ethanol, water, and pyridine, and vortexed rigorously with 300 μL phenylisothiocyanate (PITC) until clear. 50 μL of this was added to each well using the Multipette® at maximum dispensing speed. The plate was then covered and left to incubate at room temperature for 20 min, and then dried under nitrogen for one hour. Then, 300 μL of the extraction solvent was added to each well using an 8-channel pipette. The plate was then shaken for 30 min at 450 rpm (or "low speed") to ensure no spillover, and then centrifuged for 2 min at 500*g* so the fluid would pass through the upper filter plate into the capture plate.

After removing the filter plate, 10 μL of the extracts were transferred into the other 96 deep well plate for FIA and diluted with 490 μL FIA Solvent. The two deep well plates were then covered with airtight silicon mats and shaken for 10 min at 600 rpm. The LC-MS system was equilibrated with starting condition: 100% FIA Solvent, with a flow rate of 30 μL/min at 50° C with 20 μL injection volume. Retention time windows were manually adjusted, and technical validity was assessed in MetIDQ™ software and using Sciex Analyst®.

Biocrates' add-on software tool Metabo*INDICATOR*™ also provides information on up to an additional 230 biologically relevant metabolite sums and ratios associated with biochemical pathways. The analysis of sums and ratios of quantified metabolites may reveal patterns and disruptions of relative proportions that were not apparent by the individual metabolite concentrations.

2.2.2.2. Cytokine profiling. Considering the central role of inflammation in the POPs-endometriosis link, we extended the molecular profiling with a panel of inflammatory cytokines (Interleukin-6, −8, MCP-1 and TNF-α) analysed for a subset of samples using 4-PLEX technology in the Center for Immuno Monitoring Nantes Atlantic (CINMA) platform under ISO9001 and ISO15189 standards.

2.3. Data analysis

Demographic characteristics were summarised using mean and standard deviation (SD), as well as median and interquartile range (IQ) for continuous variables and frequency and percentage for categorical variables. Statistical comparison between groups (i.e. controls, cases without endometrioma (noOMA), and cases with endometrioma (OMA)) was performed using Kruskal-Wallis and Mann-Whitney Tests for continuous variables and Fisher's exact test for categorical variables.

Data analysis encompassed three principle sections (Fig. 1). First, each data block (i.e. exposome [1.a], metabolome [1.b]) was analysed separately with respect to endometriosis status as the outcome. Then, the associations linking the blocks (interblock correlation) were explored irrespective of endometriosis status. Lastly, the data blocks were integrated all together to analyse their combined associations against the outcome.

2.3.1. Single-block models

2.3.1.1. POPs-endometriosis models (Path 1a)

2.3.1.1.1. Data exploration and pre-processing. Pre-processing and exploration of POPs data was conducted with a similar approach described for metabolome, integrating specific models for the assessment of joint effects of mixtures. Bivariate correlation analysis was measured using the Spearman's rank correlation. Principal Components Analysis (PCA) and Latent Variable Clustering (LVC) analysis were carried out to visualise the structure of the data and identify potential outliers (R packages *FactoMineR* and *ClustVarLV*, respectively) (Vigneau et al. 2015). Distribution analysis included inspection of distribution histograms and conducting Shapiro–Wilk tests. Exposure data was log transformed, scaled to the standard-deviation and mean centred. Selection of confounding variables, age and BMI, was conducted *a priori* based on the evidence supporting their association with POP internal levels and endometriosis risk (Cano-Sancho et al. 2019). Breastfeeding and parity were not considered confounding variables because their causal role in the pathway between POPs and endometriosis.

2.3.1.1.2. Variable selection models. Considering the high correlation of POP data and "a priori" unknown link function with endometriosis risk, we first conducted a variable selection step with three complementary methods (Lazarevic et al. 2020; Lenters et al. 2018). ENET was performed with the '*glmnet*' package, with 100x repeated 5-fold cross validation. Covariates age and BMI were set as fixed variables by setting *penalty.factor* to 0. Gradient Boosting Machine (GBM) (R package '*dismo*') was conducted using with 100 bootstrap replications for robustness considering the parameters: number of iterations = 10000, number of trees = 5, tree complexity = 2, step size = 1, learning rate = 0.01 and bag fraction = 0.8. Bayesian Kernel Machine Regression (BKMR) was conducted with R package '*bkmr*' using the probit (binomial) link function in compound-wise variable selection mode considering default *prior* parameters and 10,000 iterations to ensure convergence (Bobb et al. 2015). To guarantee robustness, we replicated 100 times BKMR models with different seeds and then, mean (SD) posterior inclusion probabilities (PIPs), between 0 (never included) and 1 (always included), were used as measure of variable importance for each variable across the replicates. Variables retained by at least 2 models were finally retained for subsequent analysis, including single pollutant models with multivariate logistic regression (MLR), refined BKMR models and integrative multiblock models.

2.3.1.1.3. Joint effects of chemical mixtures. For the subset of chemicals consistently identified through the variable selection process, BKMR was further used to examine the joint effects of the chemical ensemble, and any interactions between the chemicals, and their associations with endometriosis status. The exposure–response function (h) was modelled using a kernel machine regression that captures complex, non-linear and non-additive, exposure–response relationships, adjusting for covariates age and BMI (Bobb et al. 2015). Default *prior* parameters and 10,000 iterations were considered to ensure convergence.

2.3.1.1.4. Pooled analysis with EndoTox study. In order to increase statistical power, we combined the data from EndoxOmics- β study with data from our previous analysis of EndoTox conducted in the same

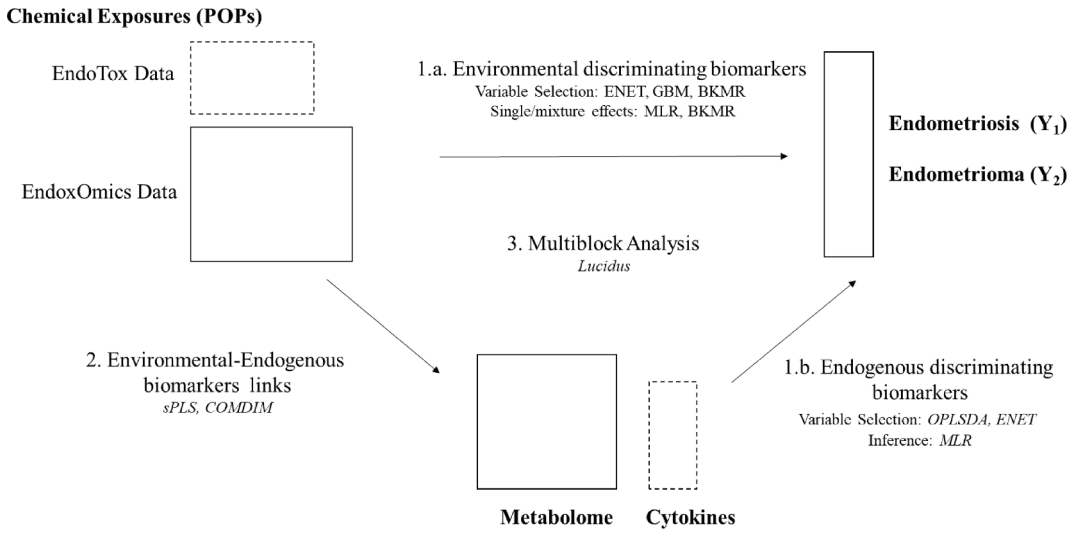


Fig. 1. Statistical workflow diagram. Path 1a and 1b (Single Block) focuses on the associations between each individual block and the outcome (Y). Path 2 (Interblock) focuses on the correlation between the exposome (E) and the metabolome (M). Lastly, Path 3 (Multiblock) integrates both E and M blocks in relation to Y. Boxes with doted borders depict datasets with incomplete observations (e.g. Cytokines) or biomarkers (e.g. EndoTox Data) for the pooled analysis. Methods used in each path are identified with abbreviations in Italics: BKMR, Bayesian Kernel Machine Regression; ENET, elastic-net regression; COMDIM, common dimension analysis; GBM, gradient boosting-machine; LUCIDus, latent unknown clustering with integrated data; MLR, multivariate logistic regression; OPLS, orthogonal partial least squares; sPLS, sparse partial least squares.

clinical setting and the same analytical platform previously reported and published elsewhere (Cano-Sancho et al. 2018; Ploteau et al. 2016; Ploteau et al. 2017). Briefly, the additional data comes from a case-control study (EndoTox) involving 99 women (55 cases, 44 controls), recruited from the same setting (Gynecology-Obstetrics Department of CHU-Nantes) during 2013–2015 resulting a final pool of 186 women. Endometriosis status was diagnosed via surgical confirmation by the same surgeon using the same clinical protocol. Internal exposure levels of target POPs were quantified in serum and adipose tissue with same methods described above (Ploteau et al. 2016; Ploteau et al. 2017). In order to account any variability between collection periods we included the study cohort as covariate in the models. Demographic distributions of this combined dataset are available in Table S1.

2.3.1.2. Metabolome-endometriosis models (Path 1.b)

2.3.1.2.1. Data pre-processing. Metabolites retained for statistical analysis were first selected on the basis of a stringent quality assessment based on internal validity (coefficient of variation $< 20\%$) and detection rate $> 75\%$. Selected metabolites as well as numerical covariates age and body mass index (BMI) were log transformed to approximate normal distributions, then centred and scaled. Normality was assessed for each data block using the Shapiro-Wilk test. Variables with non-logarithmic distributions were transformed using the package '*bestNormalize*' in R, which estimates the optimal normalisation technique (including Yeo-Johnson or Box Cox, among others). Batch-effect was corrected beforehand using *NOREVA* package (Li et al. 2017).

Variability of metabolome due to confounders like age and BMI was assessed using PCA. In order to account for related variability, two approaches were considered in all models involving the metabolome. The first approach considered the confounders in the models as covariates, and the second, a two-stage residual inclusion (2SRI) estimation beforehand, wherein models were run on the residuals after accounting for the variability explained by the covariates (Terza et al. 2008). A linear regression model was fitted to each chemical independently, adjusting for continuous variables age and BMI, and the residuals for each chemical were retained for subsequent analysis.

2.3.1.2.2. Variable selection and regression models. A supervised variable selection step was introduced to reduce data dimensionality of metabolites data, removing redundant variables and identifying the most informative metabolites using two methods ENET logistic regression and orthogonal partial least squares discriminating analysis (OPLS-DA). ENET was conducted as described above with R '*glmnet*' package, using 10-fold cross-validation for tuning the parameters in 'binomial' mode. OPLS-DA was performed using the R Bioconductor '*ropls*" package considering a cross-validation with 7 segments and 20 random permutations. Overlapping selected metabolites from ENET (with non-zero coefficients) and metabolites retained by OPLS/DA with variable importance in projection (VIP) above 1.5 were retained for multivariate logistic regression models (MLR) and integrative multiblock models. Herein, MLR was used to assess the associations between each individual chemical exposure and endometriosis, accounting for age and BMI.

2.3.2. Inter-block analysis to identify links between POPs and metabolome (Path 2)

Associations between exposure and metabolomic profiles were explored by maximizing their variance–covariance, using sparse partial least-squares regression (sPLS) within R '*mixOmics*' package. This approach allows modelling the high dimensional structures to identify the main contributors within a matrix (e.g. POPs) on the prediction of another (e.g. Metabolome) based on latent projection methods (Jain et al. 2018). The method was complemented with another multiblock method called Common Dimension (ComDim) which aims at exhibiting simultaneously common components among the various blocks and specific weights highlighting the importance of the dimension for each block (Cariou et al. 2018).

2.3.3. Multi block integrative analysis (Path 3)

Latent Unknown Clustering with Integrated Data (LUCID) approach (R '*LUCIDus*' Package) (Peng et al. 2019) was used to gain insight about the joint signature of biomarkers (POPs, metabolome and cytokine) underlying the discrimination of endometriosis subgroups. Briefly, the method integrates two main blocks of variables, (e.g. exposure variables and endogenous biomarkers) under a predefined causal structure, accounting for confounding variables to estimate a joint distribution through latent clustering. The parameters of the model and the posterior probability of being assigned to the latent clusters are estimated via the expectation–maximization algorithm, accommodating high-dimensional and collinear data via regularization. The number of

clusters K were identified using the Bayesian Information Criteria, without penalty parameters since the subset of variables underwent a comprehensive variable selection process.

2.3.4. Software

All statistical analyses were performed using R version 4.0.3. Pathway enrichment analysis was further performed with MetaboAnalyst v 5.0 in order to identify metabolic pathways which may potentially be associated with endometriosis and/or POPs.

3. Results

3.1. Population characteristics

Participant demographic data are displayed in Table 1. Neither age nor BMI significantly differed across groups of endometriosis status (Kruskal-Wallis test, $p = 0.29$ and 0.47, respectively). Approximately half (44%) of the participants were never smokers; smoking status did not significantly differ across groups (Fisher's test, $p = 0.75$). Of the 43 participants who reported use of hormonal contraception (e.g. combined oral contraceptives, progesterone only pills, vaginal ring, hormonal intrauterine devices), most (n = 35) used for 8 or more years, which was significant across the groups (Fisher's test, $p < 0.05$). Data on non-hormonal contraception use (e.g. condoms, copper intrauterine devices) was collected but not considered to be a potential confounder. Fewer than half of the women had become pregnant (45%), given birth (35%), or breastfed for any length of time (17%). In turn the control group had lower levels of IL-6 and IL-8.

3.2. Associations between POPs exposure with endometriosis status (Path 1.a)

Concentrations of seven PFAs, eight DL PCBs, six NDL PCBs, and six OCPs were quantified in>75 % of participants and retained for statistical analysis. Distribution of POPs and cytokine levels in serum are summarized in Supplemental Table S2 showing that endometriosis groups had significantly larger levels of PCB 157, sum of TEQ PCBs and pesticide *trans*-nonachlor ($p < 0.001$), compared to the control group. Bivariate analysis using Spearman's rank correlation revealed generally high correlations within chemical families specially for PCBs (Fig. 2). Correlations between the concentrations of the chemicals were higher amongst controls than cases, with little difference between cases OMA and noOMA (Fig. S1). Latent clustering analysis revealed 2 main clusters within the exposure data, mostly corresponding with major exposure families with structural similarity, PFAS and organochlorinated chemicals (Fig. S3.A and S3.B). Only 58.7% of the variability could be explained by the first two components (Dim1 46%, Dim2 12.7%) from PCA (Fig. S3.C) with high overlapping of individual observations projected onto the first 2 components (Fig. S3.D).

3.2.1. Variable selection and single pollutant models

Ranking of variables computed from the three variable selection models are presented in Fig. 3 in descending order of specific model metrics including variable importance in case of ENET (Fig. 3A), contribution to the model fit for GBM (Fig. 3B) and PIPs in case of BKMR (Fig. 3B). For practical interpretation, ranking of variables from BKMR based on PIPs provide an ordering of exposure based on variable importance similar to GBM contribution or ENET, yet no shrinkage of coefficients to zero is conducted and a threshold must be defined

Table 1
Participant demographic characteristics of 87 participants enrolled within EndoxOmics-β study. Distributions between groups were compared statistically using Kruskal-Wallis test for continuous variables, and Fisher's exact test for categorical variables. Abbreviations: IL, interleukin; IQR, interquartile range; MCP, Monocyte Chemoattractant Protein; noOMA, deep endometriosis without endometrioma; OMA, deep endometriosis with endometrioma; TNF, tumor necrosis factor.

	Control* (N = 12)	NoOMA (N = 26)	OMA (N = 49)	p-value
Age (years)				0.288
Mean (SD)	32.5 (2.92)	34.6 (6.15)	34.2 (4.79)	
Median [Min, Max]	32.4 [27.6, 35.9]	35.0 [24.0, 48.0]	34.0 [26.0, 47.0]	
BMI (kg/m^2)				0.466
Mean (SD)	22.7 (2.53)	22.9 (3.94)	24.1 (3.75)	
Median [Min, Max]	22.1 [18.3, 27.0]	21.9 [16.9, 31.2]	23.4 [16.7, 32.1]	
Smoking Status				0.754
Never	7 (58.3%)	12 (46.2%)	19 (38.8%)	
Ever	4 (33.3%)	10 (38.5%)	19 (38.8%)	
NA	1 (8.3%)	4 (15.4%)	11 (22.4%)	
Hormonal Contraceptive Use				0.044
0	0 (0%)	3 (11.5%)	4 (8.2%)	
0–7 yr	2 (16.7%)	4 (15.4%)	2 (4.1%)	
8–10 yr	5 (41.7%)	6 (23.1%)	13 (26.5%)	
> 10 yr	4 (33.3%)	2 (7.7%)	5 (10.2%)	
NA	1 (8.3%)	11 (42.3%)	25 (51.0%)	
Parity				0.715
0	7 (58.3%)	18 (69.2%)	26 (53.1%)	
1	2 (16.7%)	4 (15.4%)	10 (20.4%)	
2+	2 (16.7%)	4 (15.4%)	8 (16.3%)	
NA	1 (8.3%)	0 (0%)	5 (10.2%)	
Breastfeeding				0.842
0	9 (75.0%)	18 (69.2%)	31 (63.3%)	
< 3 mo	2 (16.7%)	3 (11.5%)	4 (8.2%)	
> 3 mo	0 (0%)	1 (3.8%)	5 (10.2%)	
NA	1 (8.3%)	4 (15.4%)	9 (18.4%)	
Serum lipids (g/dL)				
Median (IQR)	0.52 (0.46–0.62)	0.50 (0.42–0.57)	0.51 (0.48–0.57)	0.56
Cytokine profiles (pg/mL)	Missing (N = 0)	Missing (N = 2)	Missing (N = 22)	
IL-6	0.00 (0.00–0.00)	0.90 (0.00–6.10)	1.79 (0.00–7.96)	0.010
IL-8	4.10 (2.26–6.91)	16.59 (6.39–278.25)	9.04 (5.45–75.74)	0.017
MCP-1	258.26 (233.46–351.83)	350.29 (272.67–561.88)	343.91 (288.75–675.37)	0.091
TNF-alpha	2.47 (0.00–5.88)	4.13 (2.25–6.67)	3.84 (0.02–7.73)	0.47

* Control group were women attending surgery for tubal ligation or fertile women undergoing assisted reproductive technology for male infertility or oocyte donation. Fibroids or polycystic ovarian syndrome were not eligible.

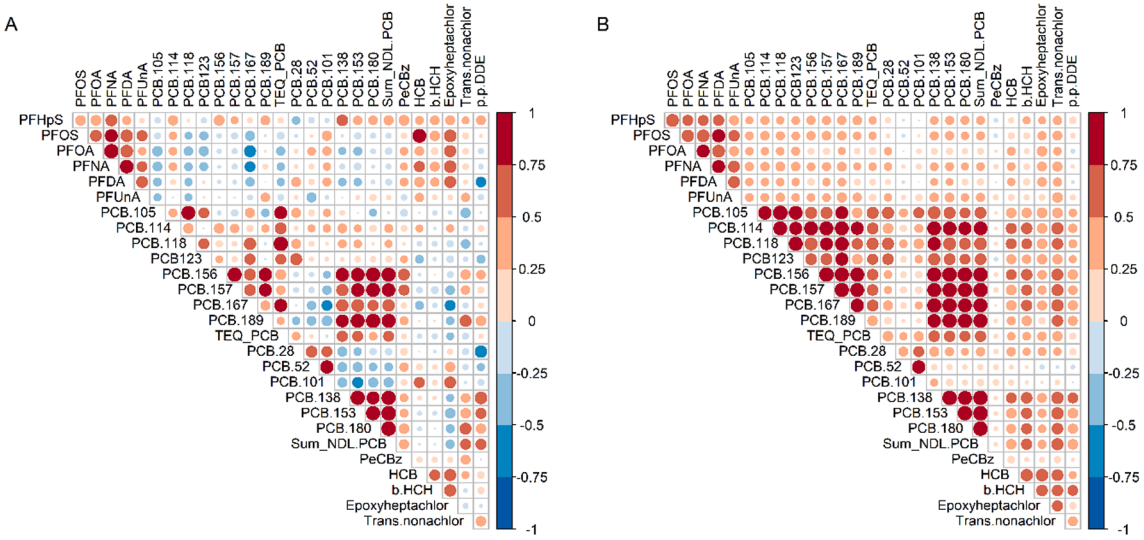


Fig. 2. Spearman's rank correlation heatmaps among serum concentrations of 29 biomarkers of exposure to POPs among controls (A) and cases (B).

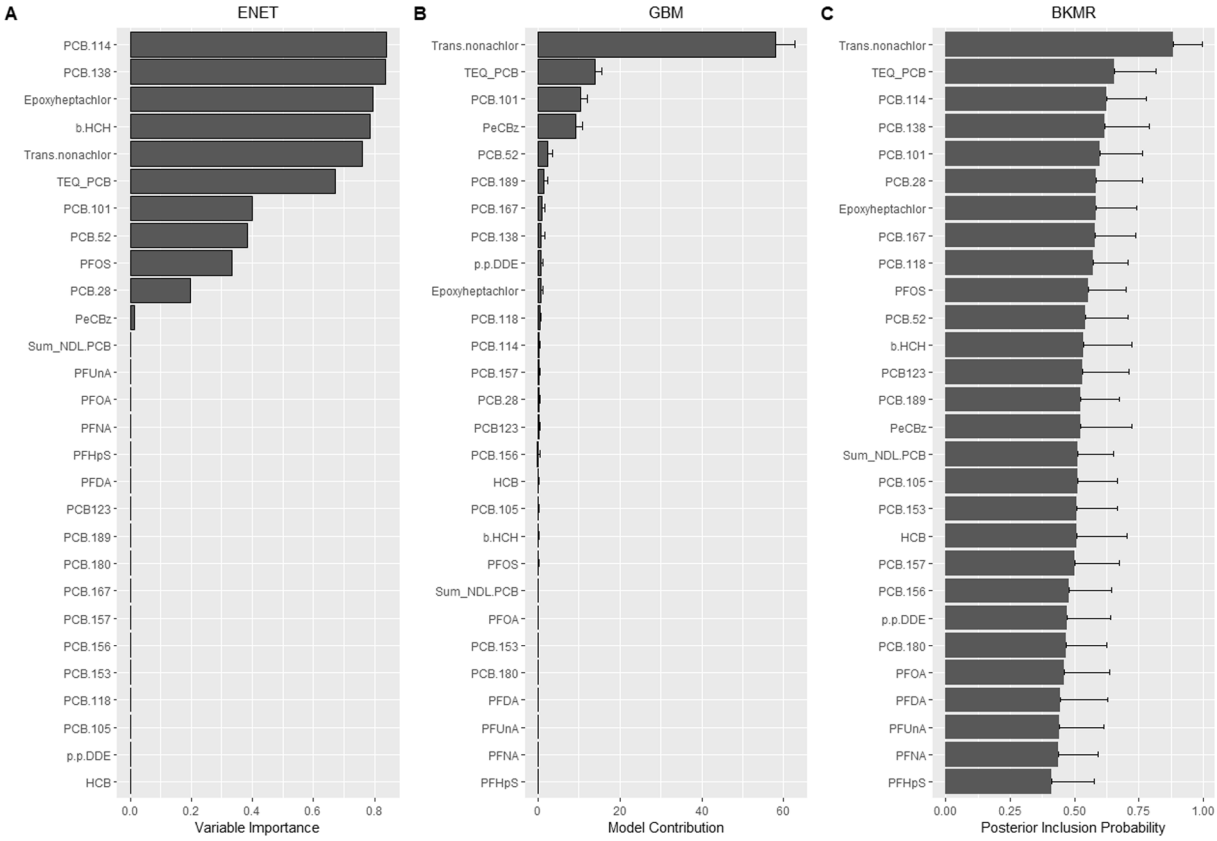


Fig. 3. Variable importance rankings measuring the association between individual exposure marker levels measured in serum and endometriosis status using (A) Elastic-net (ENET) - Data is presented as weighted non-zero coefficients of selected variables following 100x repeated 5-fold cross validation, (B) Gradient Boosting Machine (GBM) - Data are presented as the distribution (mean ± SD) of the scaled percentage contribution to the model following 100-fold replications, and (C) Bayesian Kernel Machine Regression (BKMR) - Data are presented as the distribution (mean ± SD) of the Posterior Inclusion Probabilities (PIPs) following of 10,000 iterations and replicated 100 times. All models considered age and BMI as covariates.

(Lazarevic et al. 2020). Top ranked chemicals by GBM and BKMR were consistent *trans*-nonachlor and Toxic Equivalents (TEQ) for dioxin-like PCB (TEQ-PCBs), with slight differences with ENET. Nonetheless, the retained biomarkers were quite consistent across models irrespectively of minor differences of ordering, with major presence of non-dioxin-like PCBs and pesticides (Table S3). Conversely, small associations were found between most PFAS and endometriosis, with the exception of PFOS that ranked high with ENET and BKMR. Results from GBM, expressed as variable importance (Fig. 3B), showed that *trans*-nonachlor (mean 43.6 ± 3.9) singly outranked all other chemicals, with a mean over fourfold the next most contributory variables. The profile of PIPs from BKMR showed larger probabilities for *trans*-nonachlor with

smoother differences between the rest of chemicals, challenging the variable selection procedure. We observed some instability of PIPs for most chemicals with the exception of *trans*-nonachlor, motivating us the replication of models 100 times to gain robustness. In order to support the refinement of subsequent models we considered a threshold of PIP of 0.55 allowing a balance between model complexity and variance. The three models replicated on the residuals of the 2SRI analysis did not significantly alter the results of the variable selection (Results not shown).

Retained POPs in the variable selection step were modelled with MLR in order compute inferential statistics with minor impact of false discovery rates. Results from MLR are summarized in Fig. 4A for the estimates from EndoxOmics (n = 87) and Fig. 4B from the pooled analysis with EndoTox (n = 186). Overall the results from both analyses were quite consistent, being *trans*-nonachlor the most relevant exposure biomarker positively associated with overall endometriosis risk followed by TEQ PCB in case of EndoxOmics and PCB 114 in the pooled analysis. Globally, the pooled analysis provided robustness and precision of estimates with narrower confidence intervals and larger statistical significance. The statistically significant biomarkers were *trans*-nonachlor, OR (95% CI) 3.38 (2.06–5.98), $p < 0.0001$; PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$; PCB 118 OR (95% CI) 1.6 (1.08–2.42), $p = 0.02$ and PCB 167 OR (95% CI) 1.64 (1.02–2.66), $p = 0.04$. In turn PCB 28 was statistically negatively associated with endometriosis risk OR (95% CI) 0.69 (0.48–0.98), $p = 0.04$.

3.2.2. *Joint effects of chemical mixtures*

The refined model mixture model was built with the top 11 chemicals with larger PIPs in the BKMR variable selection step (Fig. 3C) and the results are summarized in Fig. 5. The trends of the exposure–response functions h(z) for each of the 11 included chemicals are shown in Fig. S4A, which can be interpreted as the relationship between chemicals and a latent continuous marker of endometriosis status when all other chemicals are held at their median levels. Trans-nonachlor exhibited a non-monotonic inverted-U trend in its associations with endometriosis. PCB 114 and TEQ-PCBs showed the steeper positive associations whereas PCB 28, 101 and 138 the negative ones.

Fig. S4B depicts the overall risk summary between the combined exposures and endometriosis status as a continuous link function. The overall risk summary followed a flattened S-curve, suggesting a slightly non-monotonic increase in overall risk with increasing percentiles of exposure to the mixture of this chemical subset, but not significantly (credible intervals overlapping the null). Individual variable contributions to the overall risk are depicted in Fig. S4C when the rest of the mixture was fixed at the percentiles 25, 50 and 75. Though no individual variable's risk contributions were statistically significant, there were some observable trends. Most notably, *trans*-nonachlor had an increasing and overall positive contribution to the risk associated with the endometriosis outcome with increasing quartiles that would suggest a potential interaction. Conversely, PCB 28 showed a decreasing and overall negative risk contribution with increasing quartiles of the mixture. We further explored the exposure–response function of each chemical while holding a second predictor at fixed quantiles to explore potential two-way interactions of exposures (Fig. S5). Analyses indicated the presence of a non-linear relationship between exposure to pesticides *trans*-nonachlor and endometriosis risk. Two-way synergic interactions were suggested between *trans*-nonachlor and PCB 101, 138 and 167, which exhibited more striking effects in the higher quantiles of *trans*-nonachlor.

The results from the pooled analysis with EndoTox data are depicted in Fig. 5. Slight differences were noticed in the variable selection step with pooled data, for instance the pesticide p,p'-DDE was retained instead of Epoxy-heptachlor, whereas TEQ-PCBs did not exhibit larger PIPs supporting the inclusion in the refined model. The individual trends of PCB 114, 167 and Trans-nonachlor were similarly positive, whereas PCB 28 was also negatively related with endometriosis risk (Fig. 5.A). Interestingly, the cumulative effect was steeper in the pooled analysis showing credible intervals almost above the null in the higher quantiles (Fig. 5.B). Individual variable contributions to the overall risk did not reveal interactive trends (Fig. 5.C), however did show that *trans*-nonachlor has a contribution to the overall risk since the estimates fall above the null in any of fixed quantiles.

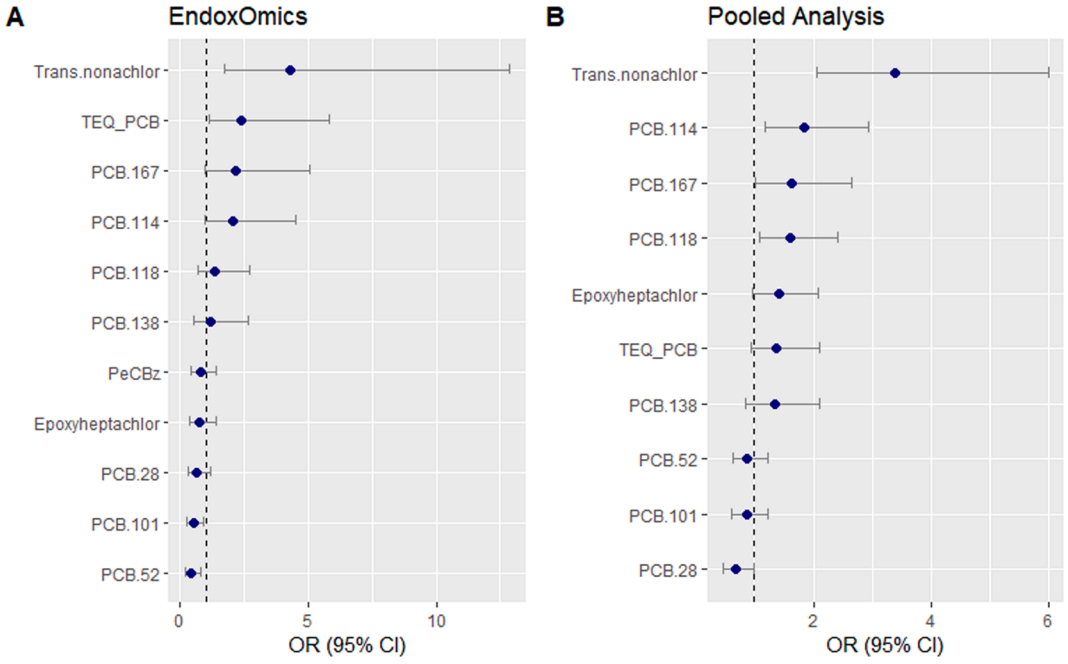


Fig. 4. Forest plot depicting the association (Odds Ratios [OR] and 95% confidence intervals) between individual exposure marker levels measured in serum and overall endometriosis risk in the EndoxOmics study (A) and pooled analysis with Endotox study (B) measured by multivariate logistic regression, adjusted by age and body mass index and study (EndoxOmics/EndoTox). The subset of POPs was identified by at least 2 models in the variable selection step (Table S3).

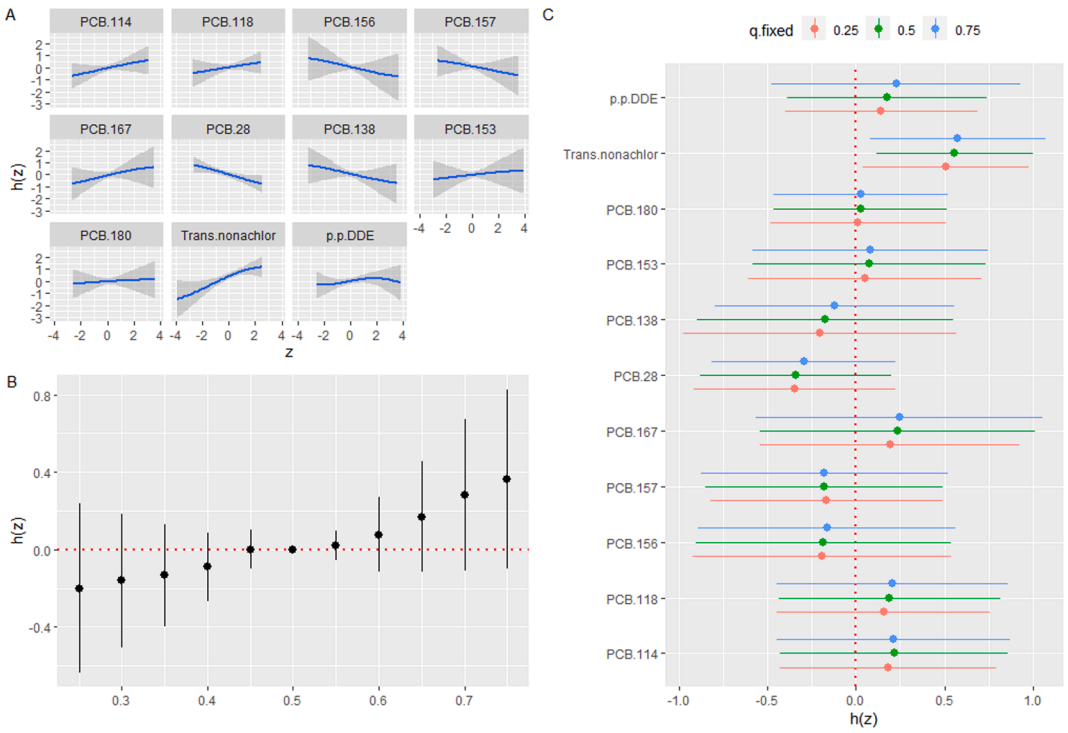


Fig. 5. Pollutant mixture analysis of endometriosis risk with the pooled EndoxOmics and EndoTox studies using BKMR depicted through (A) univariate exposure–response for each chemical (blue line) with 95% CI (grey silhouette) between the selection of variables and endometriosis. h(Z) can be interpreted as the relationship between chemicals and a latent continuous marker of endometriosis status, (B) joint effect overall risk summary of exposures on endometriosis by BKMR model when all chemicals at certain percentiles are compared to all other chemicals at the 50th percentile, and (C) single variable risk summary of each chemical by quartiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Associations between POPs exposure with endometriosis status (Path 1.a)

3.3.1. Exploratory analysis

After stringent quality assessment, 352 lipids quantified across ten families were retained: acylcarnitines (n = 33), ceramides (n = 13), cholesterol esters (n = 16), diacylglycerols (n = 12), dihydroceramides (n = 1), glycerophospholipids (n = 87), glycosylceramides (n = 10), sphingolipids (n = 14), sugars (n = 1), and triacylglycerols (n = 165). Lipids were found to be highly correlated, especially within families (Fig. 6). 37 functional metabolite ratios were also quantified. A complete list of metabolites quantified can be found in Table S4.

3.3.2. Variable selection and regression.

Variable selection of metabolomic data conducted with ENET and OPLS-DA showed an overlapping of 26 top metabolites retained for MLR and multiblock analysis (Fig. 7A and B). Despite the order of metabolites was modified between methods, we found consistent identification of most relevant features. In any case, the discrimination capacity of OPLS-DA model remained low, suggesting little dysregulation of metabolome due to endometriosis status (Fig. 7C). Selected metabolites were further analyzed with MLR adjusting for age and BMI (Fig. 7D), showing that 8 metabolites out of 26 were statistically significant ($p < 0.05$) and other 8 metabolites with marginal significance ($p < 0.1$). These included the functional ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio.12aOH.BAs.to.Non12aOH.Bas), ratio of diglycerides to triglycerides (Ratio.DGs.to.TGs) or ratio sphingomyelins to phosphatidylcholines (Ratio.SMs.to.PCs), with respective OR (95 %, p-value) of 3.12 (1.46–7.93, p = 0.007), 3.10 (1.42–7.99; p = 0.009) and 2.54 (1.29–5.68, p = 0.012) (see Fig. 7D).

3.4. Associations between POPs exposure and metabolome profiles (Path 2a)

The associations between POPs and metabolic profiles were globally small and consistent across two approaches (ComDim and sPLS). Despite differences in number of components (3 in ComDim and 2 in sPLS), we found substantial overlapping of most relevant features retained from each data block. For instance, in both cases, a similar metabolic congener profile was related with one component associated with PCBs (related to triglycerides) and another with PFAS, highlighted by the presence of tetradecenoylcarnitine (C14:1) and hexosylceramide (HexCer) d18:2/24:0, among others (Fig. S7 for ComDim results and Fig. S8 for sPLS results). Specific findings were also highlighted by each approach, for instance ComDim revealed the links between PFOA, PeCBz and HCB in component 2 with a list of functional ratios, including the ratio of diacyl-hosphatidylcholines and cyl-alkyl-phosphatidylcholines to choline (Fig. S6D). In turn sPLS approach showed the central position of the ratio of hydroxylated sphingomyelins to non-hydroxylated sphingomyelins (Ratio.SMOHs.to.SMNonOHs) and HexCer 18:1/24:0 in the associations with PCBs and PFAs.

3.5. Associations between POPs exposures and cytokine profiles (Path 2b)

Summary results from analysis of associations between POP exposures and inflammatory status can be found in Supplemental Fig. S8. In a first step we attempted to discriminate the inflammation profiles (High vs Low) dichotomizing the levels of IL-8 based on the POP signature using PLSDA, showing moderate/low discrimination being *p,p*'DDE, PFHpS, TEQ-PCBs and b-HCH the congeners that scored with higher VIPs (Fig. S8.A and S8.B). The chemicals with higher VIPs (VIP > 1.25) were further modelled against IL-8 levels using multivariate linear regression adjusted by age, BMI and endometriosis status. The regression analysis confirmed the positive and statistically significant

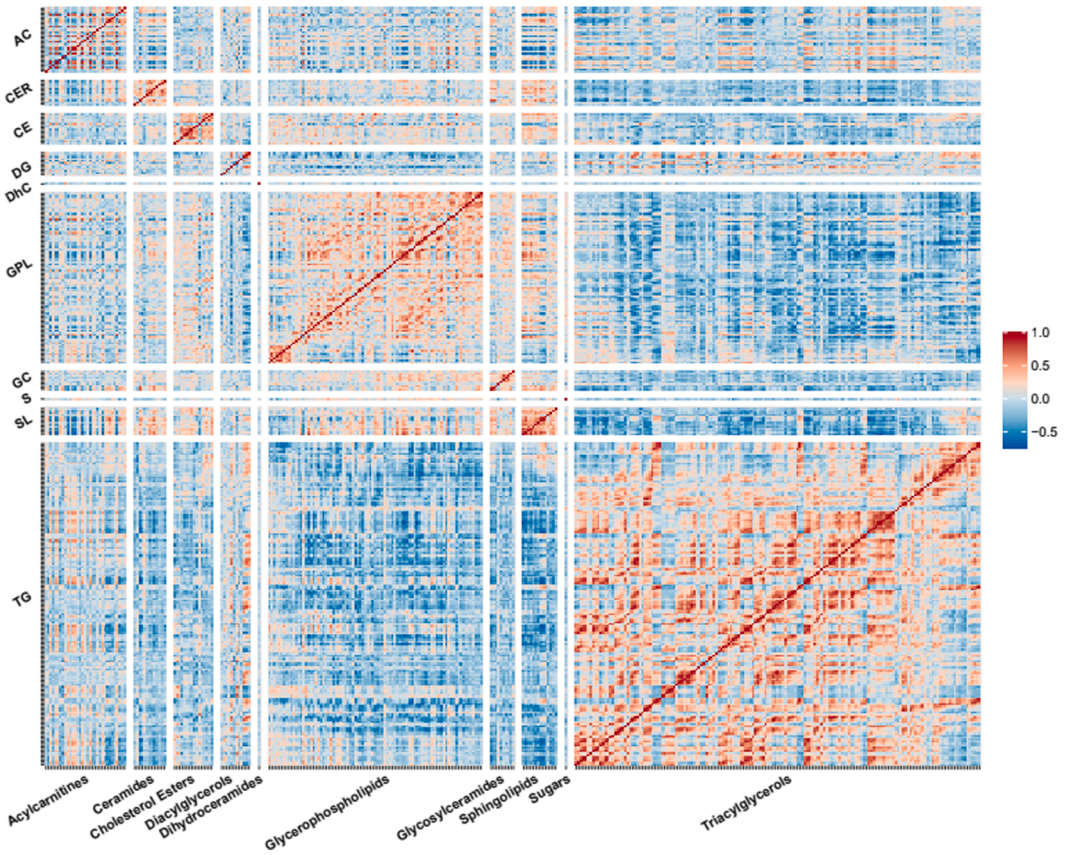


Fig. 6. Bivariate Spearman Correlations among serum concentrations of 354 lipid biomarkers, presented as $-1 < \rho < 1$. Complete list of lipids in Table S4. Abbreviations: Acylcarnitines (AC); Ceramides (CER); Cholesterol Esters (CE); Diacylglycerols (DG); Dihydroceramides (DhC); Glycerophospholipids (GPL); Glycosylceramides (GC); Sphingolipids (SL); Sugars (S); Triacylglycerols (TG).

associations between PFHpS and levels of IL-8, β (95 % CI) 0.26 (0.05–0.48), $p = 0.019$. The rest of POPs showed in general null results. In the sensitivity analysis, adjustment by endometriosis status did not exhibit a large impact on the estimates for PFHpS, regardless of considering two (cases vs controls) or three levels (OMA, noOMA vs controls). Nonetheless, for TEQ-PCBs and *p,p*'DDE the adjustment for endometriosis status notably reduced the magnitude coefficients (over 10%).

3.6. Integrated multiblock analysis (Path 3)

This analysis was conducted with the subset of 11 POPs and 26 metabolites selected in Path 1a and 1b respectively, identifying two main latent clusters (*K*) on the joint discrimination model (Fig. S9). Latent Cluster 2 (LC2) was associated with a higher risk (OR = 1.51) of endometriosis compared with the referent Cluster 1. POPs showed a major probability of being assigned to the high risk cluster LC2, being *trans*-nonachlor (OR = 1.88), PCB 167 (OR = 4.08) and PCBs 52 (OR = 2.08) the chemicals with larger positive ORs, whereas PCB 28 (OR = 0.83) and 138 (OR = 0.13) the ones with larger negative probabilities. High risk LC2 was also characterised by overall lower levels of most triglycerides (TG), lysophosphatidylcholine (lysoPC) a C20:4, ceramide (d18:1/18:0), ratio of phosphatidylcolines PCs to choline and the ratio of TG to fatty acids. In turn, LC2 had larger levels of (PCs), ratio SM to PCs, ratio DG to TG and ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio.12aOH.BAs.to.Non12aOH.BAs).

In a secondary analysis, for a subset of samples (n = 67) with available quantitative cytokine profiles, we extended the integrative analysis and considered different endometriosis outcome groupings. This analysis was refined in order to include the most relevant variables identified in the variable step procedure, resulting in a subset of 6 POPs (Table S3), 8 metabolites and ratios and 2 cytokines. The results for overall endometriosis risk showed poor performance with a latent cluster (LC2) more associated with POPs but little association the outcome. More performant results were found for OMA, in terms of model convergence (Fig. 8). In this case, LC2 was also more positively associated with POPs including PCB 114, TEQ-PCBs and *trans*-nonachlor and negatively associated with PCB 101, PCB 138 and epoxyheptachlor. In turn, LC2 showed higher level of IL8 and MCP1 and the ratio of SMs to PCs, ratio DGs to TGs and ratio 12aOH.BAs to Non12aOH.BAs (Fig. 8). Whereas cytokines showed little contribution to clustering when the model was built for total cases vs controls; they contributed substantially in discriminating the OMA group from the combined group of controls with noOMA.

4. Discussion

To our knowledge, this is the first study investigating the associations between mixtures of POPs and endometriosis by integrating quantitative metabolomics and cytokine profiling in order to gain insight on the potential underlying biological mechanisms. We have also developed a comprehensive data analysis framework favouring the efficient integration of targeted biomarker data generated by multiple high-resolution MS platforms. Despite the modest sample size of the study, we identified several organochlorine pesticides (i.e. *trans*-nonachlor or PCB 114) to be positively associated with endometriosis, as well as some non-coplanar PCBs (28 and 52) which whose roles in pollutant mixtures seems to be important. These results were consistently strengthened by a pooled statistical analysis combining data from previous study conducted in the same region under the same clinical and analytical

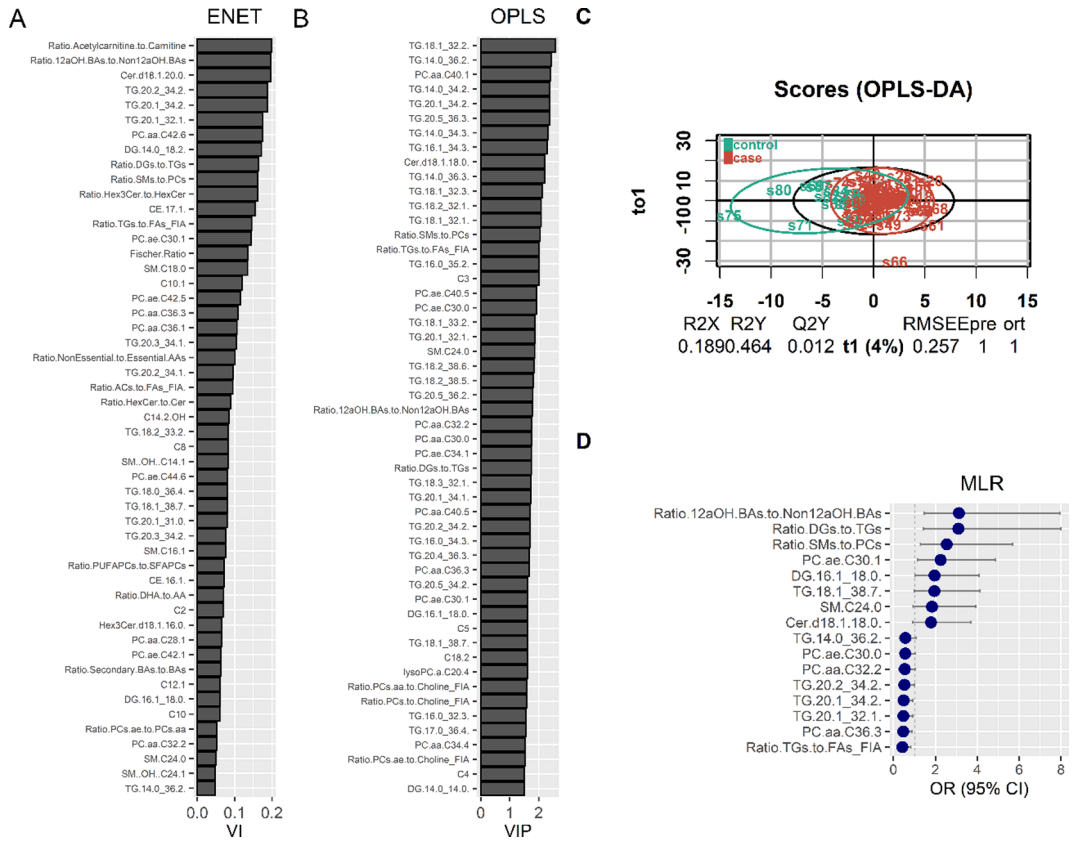


Fig. 7. Bar plot depicting the variable importance (VI) of most relevant metabolites from elastic net (ENET) logistic regression (Panel A) and variable importance on projection (VIP from orthogonal partial least square discriminating analysis (OPLS-DA, Panel B) on the discrimination of endometriosis status (Cases vs controls). Score plot from PLS-DA is shown in Panel C for cases (red) and controls (green). Forest plot in Panel D shows the odds ratios (OR) and 95% confidence intervals for the subset of overlapping metabolites selected by ENET ($\beta \neq 0$) and OPLS-DA (VIP > 1.5), estimated by Multivariate Logistic Regression (MLR), adjusted by age and body mass index. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

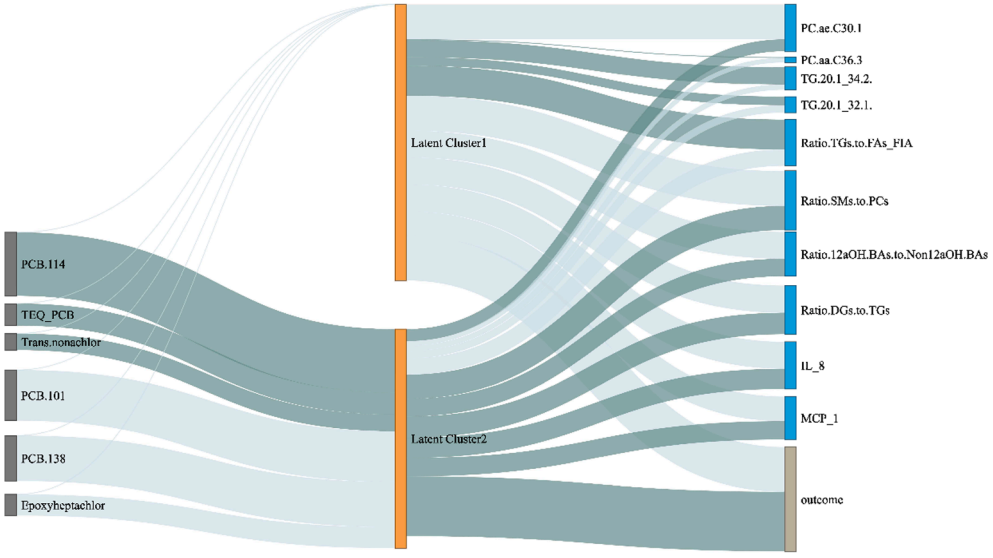


Fig. 8. Sankey diagram of the secondary structural integrated analysis of POPs exposure and individual lipid metabolites and cytokines in serum for the identification of latent variable clusters associated with severe cases of endometriosis (OMA). Dark grey bands represent positive associations and light grey bands indicate negative associations, with the width proportional to the size of the association. POPs exposures and cytokines are positively associated to Latent Cluster 2, which contributes positively towards outcome (OMA).

settings. Mixture analysis with BKMR showed that the overall mixture of selected POPs (PCBs and pesticides) had a positive trend on cumulative effects and highlighted the contribution of *trans*-nonachlor within the mixture regardless the concentrations of the others. Conversely, the mixture analysis suggested interactive synergy between *trans*-nonachlor and PCB 118 or PCB 167, strengthening the associations with endometriosis risk only at higher concentrations of the pesticide. We have also elucidated a differential metabolome between cases of endometriosis and controls, identifying a handful of lipids and functional metabolite ratios related to this discrimination including the ratio of 12-alpha-hydroxylated bile acids to non-12-alpha-hydroxylated bile acids (ratio 12aOH BAs to Non-12aOH BAs), the ratio of diglycerides to

triglycerides (Ratio DGs to TGs) and the ratio sphingomyelins to phosphatidylcholines (Ratio SMs to PCs). In turn, the integrative structural analysis revealed that patients with OMA, had a profile characterised by higher concentrations of POPs (*trans*-nonachlor, PCB 114 and TEQ-PCBs), inflammation markers (IL-8 and MCP-1) and a metabolic profile with dysregulated functions of bile acid balance and lipase activity.

The mixture profile of POPs combined historical chemicals like PCBs and OCPs with more emerging classes like PFAS, has never been explored before in relation with endometriosis risk. Serum concentrations of the seven PFAS compounds and six NDL PCBs were comparable to those found in similar populations of French women (Koual et al. 2019; Ploteau et al. 2017), as well as those reported in a recent study on a 2014–2016 French population for PFAS (Fillol et al. 2021). The POPs found to be most associated with endometriosis are consistent with our previous findings (Ploteau et al. 2017; Cano-Sancho et al. 2019). In a previous *meta*-analysis, organochlorine pesticides as a family were associated with endometriosis risk with an OR (95% CI) of 1.23 (1.13–1.36), whereas PCBs had an even stronger association with an OR (95% CI) of 1.70 (1.20–2.39) (Cano-Sancho et al. 2019). PCBs are often studied either individually, grouped based on their aryl hydrocarbon receptor affinity as DL or NDL, or as a sum of TEQ (Pauwels et al. 2001). For instance, there has been experimental evidence that DL but not NDL PCBs are linked to the endometriosis through pro-inflammatory interactions (Huang et al. 2017). Other studies have considered the estrogenic potential of PCBs as rationale for biologically-informed grouping, finding higher risk of endometriosis among women with the highest concentrations of anti-estrogenic PCB summed (Buck Louis et al. 2005). Only one recent study similarly used BKMR to analyse the adipose to serum ratio (ASR) of PCB and OCP mixtures, finding positive associations between the ASR for OCPs and estrogenic PCBs and endometriosis (Pollack et al. 2021).

Our results further support the interest of analysing POP mixtures on endometriosis risk. For instance, BKMR allowed us to characterize the cumulative patterns of mixtures of PCBs and pesticides, accounting for interactive effects. On this regard, synergy between *trans*-nonachlor and PCB 118 or PCB 167 were suggested (Fig. S5). The results from BKMR also showed that considering the co-exposure may result in different exposure–response associations to those from single pollutant models. For instance, the null associations of PCB 138 (Fig. 4) tended to be negative in BKMR, especially when the rest of mixture was set to the lowest 25th percentile (Fig. S4), yet did not reach significance. The flexibility of BKMR elucidated non-monotonic inverted U-shaped effects of *trans*-nanochlor within the EndoxOmics study. The negative associations for some PCBs (i.e. PCB 28 or PCB 52) remain still unclear, though we have noticed similar patterns in our previous EndoTox study among congeners with low chlorination. It is possible that the inconsistent results from previous studies on POPs can be explained not only by the heterogeneous nature of this group of chemicals, but also the potential presence of interactions between PCBs and other concomitant exposures may be possible. On this regard, our study also included PFAS, a family of POPs with specific physiochemical and toxicological characteristics (e.g. amphiphilic) that remains barely explored in relation with endometriosis risk. Our results did not show relevant associations of PFAS alone nor in mixtures with historical POPs; however, one previous study found positive associations between serum levels of perfluorooctanoic acid (PFOA; OR = 1.89 [95% CI = 1.17–3.06]), perfluorononanoic acid (2.20 [1.02–4.75]) and endometriosis risk (Louis et al. 2012). Future studies should consider this group of chemicals in endometriosis research due to the relevance in terms of population exposure (e.g. present in all samples from this study at relevant concentrations) and toxicological effects. We should also highlight the fact that our study was conducted with serum biomarkers of POPs which may be an analytical challenge as certain underlying biological process may alter the circulation of serum lipids (Cano-Sancho et al. 2018). In our case, we did not notice differences of circulating levels of lipids between groups, minimising any potential bias due to normalisation of biomarkers (Cano-Sancho et al. 2020). We recommend future studies to consider the analysis of POPs and endogenous metabolic markers within adipose tissue, as the stores of lipophilic contaminants in adipose tissue may more stable and accurately depict the long-term body burdens of POPs exposures (Mustieles and Arrebola 2020).

Another highlight is the targeted metabolic profiling that allowed the identification of a handful of lipids and functional ratios to be differentially regulated between endometriosis cases and controls. Specifically, women with endometriosis presented larger ratios of 12-α OH to non-12-α OH bile acids. This was especially true for the endometrioma subgroup. Bile acids are molecules with both hydrophobic and hydrophilic components which act as signalling molecules involved in lipid metabolism but also enterohepatic absorption-excretion cycle of POPs (Jandacek and Tso 2007). The 12-α-hydroxylation of BAs is mainly regulated by a liver-specific enzyme (Vlahcevic et al. 2000), and the imbalance of ratio of 12-α OH to non-12-α OH BAs has been suggested as a mechanism linking dyslipidaemia with insulin resistance, throughout the Akt-FoxO1 pathway and modulation of levels of triacylglycerols (Haeusler et al. 2013). In female mice, short term exposure to TCDD tended to decrease the levels of BA in serum and liver, including the levels 12-OH BAs throughout the suppression of 12α-hydroxylase Cyp8b1 mediated by the AhR activation (Csanaky et al. 2018). We have also found the ratio of glycerolipids diacylglycerols (DGs) to triacylglycerols (TGs), as well as of TGs to fatty acids (FAs) to be significantly different between endometriosis cases and controls. For both ratios, we found that women with endometriosis had increased lipase activity. This altered lipid profile has been found to be linked to both endometriosis and implantation status for women seeking *in vitro* fertilisation treatments (Domínguez et al. 2017; Matorras et al. 2020). We have also found larger ratios of sphingomyelins (SMs) and phosphatidylcholines (PCs) and absolute levels of some PCs (e.g PC ae C30:1) associated with endometriosis risk. Sphingomyelins (SMs) and phosphatidylcholines (PCs) have been identified as potential biomarkers for ovarian endometriosis in previous studies (Vouk et al. 2012). It is hypothesised that elevated levels of SMs and PCs may contribute to the suppression of apoptosis and alter lipid-related signalling pathways. In turn, the ratio of SMs to PCs is an indicator of SM synthesis from ceramides and was found here to be a potential predictor of endometriosis.

In addition, inflammatory cytokines were found to be more associated with OMA compared with combined group of controls and noOMA than in overall endometriosis risk (compared to controls). The integrative analysis elucidated a shared signature between POPs (dioxin-like PCBs TEQs, PCB 114 and *trans*-nonachlor) and endogenous markers of dysregulation of bile acid metabolism, lipolytic activity and inflammation were upregulated in severe endometriosis cases with endometrioma. These findings support the main underlying mechanisms elucidated in animal and *in vitro* studies, mainly reported for the prototypical toxicant TCDD (Matta et al. 2019). The contribution of each individual chemical from a complex mixture of endocrine disrupting chemicals becomes elusive in current observational research methods; however, it is likely that complex interactions may occur, especially in light of reported synergistic interactions between endogenous oestrogen (and oestrogenic chemicals) on endometriosis progression. We have also elucidated a lipotoxic profile pattern among patients with OMA and higher levels of POPs that could also suggest an interplay between POPs and lipid metabolism or adipose tissue dysfunction.

These results, however, should be taken with caution due to a number of methodological limitations, including modest statistical power due to the small sample size of this pilot study and the cross-sectional design. We attempted to address the first issue by pooling the present exposure data from this study with exposure data from our previous EndoTox study conducted in the same clinical setting and the same analytical platform (Cano-Sancho et al. 2018; Ploteau et al. 2016; Ploteau et al. 2017). Concerning the cross-sectional design, we acknowledge that a risk of reverse causation and disease progression

bias may exist. However, the stable and persistent nature of POPs supports the use of single samples (e.g. at diagnosis) as surrogate of exposures during long periods previous the collection. In any case, considering the body of evidence on endometriosis and POPs is built on cross-sectional designs (Cano-Sancho et al. 2019), future studies on endometriosis should consider prospective designs and nested case-studies to minimise the risk of bias. We should also acknowledge the statistical limitations inherent to multipollutant modelling with highly correlated data structures. This issue impairs the model performance and may easily lead to wrongly attribute the association to those exposures in the mixture with lower measurement error, despite using advanced methods as BKMR (Lazarevic et al. 2020). Nonetheless, the consistent findings across the different variable selection methods used in this study strengthened the robustness of findings yet experimental research will be required for confirmation. Conversely, the lack of statistical association for specific chemicals and/or mixture should be taken with caution in the light of these mentioned limits. Another important note is that the population controls used in this study may not be completely representative of the general population at risk for endometriosis (Upson 2020). Clinical recruitment may present different exposure and metabolic profiles than the general population, preventing the generalisation of results. Additionally, though controls comprised mostly women who presented no endometriosis symptoms, for most controls, there was no surgical or histological confirmation of absence of endometriosis due to ethical reasons. Considering that we focused only on severe stages of deep endometriosis with and without endometriosis, we believe that asymptomatic and fertile women, minimize the control selection bias. Lastly, as with all studies based on epidemiological data, finding may be subject to confounding bias. For instance, we were unable to adjust by contraceptive use due to the large proportion of missing data (37%). Nonetheless, experimental evidence suggest that interactions between estrogen and dioxin-like compounds may boost the progression of endometriotic lesions (Wang et al. 2010; Yu et al. 2008).

5. Conclusion

In summary, the present study is the first of its kind to integrate metabolomics and cytokine profiling in observational research of endometriosis to gain insight on the underlying modes of actions of POPs. This study proved the high exploratory potential of integrating multiple MS platforms simultaneously covering a comprehensive panel of endogenous and exposure biomarkers with major applications in etiological research. Using high-throughput targeted metabolomics provided a good compromise, taking advantage of the accuracy and selectivity of targeted approaches with the high analytical capacity provided by non-targeted approaches. The results, strengthened by a pooled analysis, showed positive associations between some POPs (i.e. *trans*-nonachlor OR (95% CI) 3.38 (2.06–5.98), $p < 0.0001$ and PCB 114 OR (95% CI) 1.83 (1.17–2.93), $p = 0.009$) and endometriosis risk. The integrative multiblock analysis revealed a latent cluster of women with higher risk of OMA, presenting higher concentrations of *trans*-nonachlor, PCB 114 and toxic equivalents from dioxin-like PCBs, as well as an increased inflammatory profile and a metabolic pattern characterised by dysregulation of bile acid homeostasis and lipase activity. Results suggest the role of certain POPs in promoting pro-inflammatory metabolic conditions which may be involved in the development of severe endometriosis. Our results support that further studies are required to confirm these findings, conducted on a larger and population scale designed prospectively.

CRediT authorship contribution statement

Komodo Matta: Formal analysis. **Tiphaine Lefebvre:** . **Evelyne Vigneau:** Methodology, Software, Validation. **Véronique Cariou:** Methodology, Software, Validation. **Philippe Marchand:** . **Yann Guitton:** . **Anne-Lise Royer:** . **Stéphane Ploteau:** Resources. **Bruno Le Bizec:** Resources. **Jean-Philippe Antignac:** Resources, Conceptualization. **German Cano-Sancho:** Resources, Conceptualization, Methodology, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106926.

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Associations between Exposure to Organochlorine Chemicals and Endometriosis: A Systematic Review of Experimental Studies and Integration of Epidemiological Evidence

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Background: Growing epidemiological evidence suggests that organochlorine chemicals (OCCs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), may play a role in the pathogenesis of endometriosis.

Objectives: We aimed to systematically review the experimental evidence (*in vivo* and *in vitro*) on the associations between exposure to OCCs and endometriosis-related end points.

Methods: A systematic review protocol was developed following the National Toxicology Program /Office of Health Assessment and Translation (NTP/OHAT) framework and managed within a web-based interface. *In vivo* studies designed to evaluate the impact of OCCs on the onset or progression of endometriosis and proliferation of induced endometriotic lesions were eligible. Eligible *in vitro* studies included single-cell and co-culture models to evaluate the proliferation, migration, and/or invasion of endometrial cells. We applied the search strings to PubMed, Web of Science, and Scopus®. A final search was performed on 24 June 2020. Assessment of risk of bias and the level of evidence and integration of preevaluated epidemiological evidence was conducted using NTP/OHAT framework

Results: Out of 812 total studies, 39 met the predetermined eligibility criteria (15 *in vivo*, 23 *in vitro*, and 1 both). Most studies ($n = 27$) tested TCDD and other dioxin-like chemicals. *In vivo* evidence supported TCDD's promotion of endometriosis onset and lesion growth. *In vitro* evidence supported TCDD's promotion of cell migration and invasion, but there was insufficient evidence for cell proliferation. *In vitro* evidence further supported the roles of the aryl hydrocarbon receptor and matrix metalloproteinases in mediating steroidogenic disruption and inflammatory responses. Estrogen interactions were found across studies and end points.

Conclusion: Based on the integration of a high level of animal evidence with a moderate level of epidemiological evidence, we concluded that TCDD was a known hazard for endometriosis in humans and the conclusion is supported by mechanistic *in vitro* evidence. Nonetheless, there is need for further research to fill in our gaps in understanding of the relationship between OCCs and their mixtures and endometriosis, beyond the prototypical TCDD. https://doi.org/10.1289/EHP8421

Introduction

Humans are exposed daily to complex mixtures of chemical pollutants, some of which may contribute to a disruption of endocrine functions and contribute to reproductive diseases. Chemicals with bioaccumulative properties are of particular concern, because they persist worldwide in the environment and ultimately may accumulate in human fatty tissues, despite the fact that many are now heavily regulated or banned (Pumarega et al. 2016; UNEP 2017). These chemicals include a large family of pollutants known as organochlorine chemicals (OCCs), characterized by their carbon–chlorine bonds, which comprise polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs) (Bernes 1998; Jones and De Voogt 1999). A number of OCCs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been extensively reported to interact with the aryl hydrocarbon receptor (AhR) and/or estrogen receptors and disrupt the nervous, immune, and endocrine systems at various life stages (Gore et al. 2015; Lawrence and Vorderstrasse 2013; Barouki et al. 2012).

The female reproductive tract has been shown to be especially vulnerable to the presence of hormone-disrupting chemicals, potentially leading to early puberty, infertility, adverse pregnancy outcomes, fibroids, or endometriosis (Bruner-Tran et al. 2017; Gore et al. 2015; Ho et al. 2017; Hutz et al. 2006). Endometriosis is a hormone-dependent gynecological disease characterized by the presence and growth of ectopic endometrial tissues (Zondervan et al. 2018). The precise prevalence of endometriosis in the general population is largely unknown due to difficulties in diagnosis, underreporting, and unknown prevalence among asymptomatic individuals, but estimates of prevalence range widely from 5% to 45% of individuals who menstruate (Buck Louis et al. 2011; Rawson 1991). Many gaps also remain in fully understanding the etiology of endometriosis, but it is likely multifactorial, involving genetic and environmental factors (Sourial et al. 2014; van der Linden 1996).

In a previous systematic review and meta-analysis, we synthesized the associations between OCCs and endometriosis in human epidemiological studies and evaluated the quality of the body of evidence using the comprehensive National Toxicology Program/Office of Health Assessment and Translation (NTP/OHAT) framework (Cano-Sancho et al. 2019). The overall conclusion of this review supported the existence of positive associations between exposure to PCDDs, PCBs, and OCPs and endometriosis, which is consistent with subsequently published reviews (Freger and Foster 2020; Wen et al. 2019). However, the level of evidence was

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deemed moderate, with serious risk of bias: Major methodological limitations in epidemiological research of endometriosis included the lack of population-based study designs, the inherent constraints in classifying control populations, the heterogeneity of endometriosis subtypes, the use of different OCCs biomarkers, and narrow background exposure distributions.

Associations observed from epidemiological studies, however, provide an incomplete picture and fall short of being able to provide a biological explanation for the link between OCC exposure and endometriosis. Thus, from our point of view, experimental studies are necessary to advance our understanding of the underlying molecular mechanisms and provide the support of biological plausibility to the trends observed in human epidemiological studies. The nonhuman primate model of endometriosis is considered the most reliable because it mimics the pathophysiological conditions in women (Story and Kennedy 2004; Grümmer 2006). However, ethical considerations and high economic costs have favored the development of rodent models (Bruner-Tran et al. 2018). The process of surgically inducing lesions by implanting the animal's autologous uterine horn was developed in rats (Vernon and Wilson 1985) and later adapted to mice (Cummings and Metcalf 1995a).

In addition, *ex vivo* and *in vitro* models, with primary cells and co-cultures from human biopsies, represent a straightforward way to gain insight into the molecular signaling pathways in endometrial cells (Fan 2020). To our knowledge, no studies to date are attempting to systematically gather and appraise the experimental evidence on OCC exposure and endometriosis, though some narrative reviews on the topic have been conducted (Birnbaum and Cummings 2002; Bruner-Tran and Osteen 2010; Guo et al. 2009; Rier and Foster 2003). In this context, the present work aimed to perform a systematic review applying the NTP/OHAT framework to *a*) systematically appraise experimental (*in vivo* and *in vitro*) studies reporting evidence on the associations between OCC exposure and endometriosis and *b*) to draw a conclusion based on the level of confidence of this body of evidence.

Methods

The present systematic review was conducted following the guidelines established in the NTP/OHAT handbook (NTP/OHAT 2015a), using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) principles. The protocol was registered in International Prospective Register of Systematic Reviews (PROSPERO; registration number CRD42018102618) on 17 July 17 2018, peer-reviewed, and published in January 2019 (Matta et al. 2019). Study selection, data extraction, data synthesis, and risk of bias (RoB) assessment were performed and managed using the Health Assessment Workspace Collaborative (HAWC; https://hawcproject.org/), an open-source, modular web-based content management system with a user interface (Shapiro et al. 2018). Numerical data from plots and graph images were extracted using WebPlotDigitizer (version 4.3; https://automeris.io/WebPlotDigitizer/). The RoB assessment was performed using the NTP/OHAT's RoB rating tool to assess individual study quality (NTP/OHAT 2015b), and followed the guidance of the NTP protocol for the perfluorooctanesulfonic acid/perfluorooctanoic acid (PFOS/PFOA) monograph for the integration of *in vivo* and *in vitro* data (NTP 2016). Results are reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2009).

Eligibility Criteria

The literature search strategy was initially developed by using the list of all persistent organic pollutants (POPs) determined by the Stockholm Convention (UNEP 2017) to calibrate and refine the protocol during problem formulation. Accordingly, we developed a Populations, Exposures, Comparators, Outcomes (PECO) statement (Table 1) establishing the inclusion/exclusion criteria for our systematic review. In addition, we excluded any studies that did not contain original data, such as reviews, commentaries, editorials, or conference abstracts, as well as studies not available in English. Further details of problem formulation can be found in the previously published protocol (Matta et al. 2019).

Literature Search and Study Selection

The search string was developed to identify all relevant published evidence in experimental studies (*in vivo*, *ex vivo*, or *in vitro*) with primary data on the associations between controlled exposures to OCCs and endometriosis and endometriosis-related effects by *a*) reviewing MeSH terms and literature tags used by previously identified human epidemiology studies on endometriosis for relevant and appropriate terms; *b*) adapting existing chemical lists (UNEP 2017; Wassenaar et al. 2017); and *c*) extracting several potential endometriosis-related mechanistic outcomes (Liu and Zhao 2016). The complete search string can be found in Table S1. It comprised the exposure and outcome elements of the PECO statement nested through the Boolean operators "AND/OR." A comprehensive list of 33 persistent organic pollutants identified in the Stockholm Convention with suspected endocrine-disrupting potential can be found in Table S2 (UNEP 2017). Because the scope of our review focused on OCCs, brominated congeners (i.e., decabromodiphenyl ether, hexabromobiphenyl, hexabromocyclododecane, hexabromodiphenyl ether, heptabromodiphenyl ether, tetrabromodiphenyl ether, and pentabromodiphenyl ether) and PFOS, its salts, and perfluorooctane sulfonyl fluoride were not included in the search terms. In addition to this list of chemicals, a set of OCC-related terminology was used to capture any other studies with relevant exposure. Synonyms of each chemical name were retrieved using the

Table 1. Summary of Population, Exposure, Control, Outcome (PECO) statement for *in vivo* and *in vitro* studies.

	Inclusion	Exclusion
P	*In vivo*: Experimental animal models where endometriosis can either occur spontaneously or be induced *In vitro*: Human endometrial cells or tissues	*In vivo*: Observational epidemiological studies *In vitro*: Cancer cells, nonuterine cells
E	*In vivo* and *in vitro*: • Organochlorine chemicals (OCCs)	*In vivo* and *in vitro*: • Pharmaceuticals, non-OCCs
C	*In vivo* and *in vitro*: • Has reference or control group	*In vivo* and *in vitro*: • Lacks reference or control group
O	*In vivo*: • Onset or aggravation of endometriosis • Proliferation or growth of induced endometriotic lesions • Presence-of "endometriosis-like" phenotypes with human reference standard *In vitro*: Proliferation, migration, and/or invasion of endometrial cells	*In vivo* and *in vitro*: • Outcomes unrelated to endometriosis

PubChem and ChemSpider database on 27 June 2018. An initial search was performed on 9 January 2019 in the databases PubMed/Medline (https://www.ncbi.nlm.nih.gov/pubmed), Scopus (https://www.scopus.com), and Web of Science (WoS; https://webofknowledge.com), with syntax customized for each database. A subsequent manual search of the bibliographic references of relevant articles and existing reviews was performed. A final search update was performed on 24 June 2020. No filter was applied to limit the date or language of publication during the search. The identified studies were exported to EndNote in RIS format to pool records for manual duplicate removal. Studies underwent a two-phase screening process by two independent researchers (K.M. and G.C.S.), based on the predefined inclusion and exclusion criteria of the PECO statement (Table 1). Phase I Screening was based on title and abstracts, and Phase II Screening was based on full text for the studies not excluded in Phase I. Any discrepancies were resolved by consensus or with the opinion of a third researcher (J.P.A.).

Data Extraction

Eligible studies underwent data extraction within the HAWC module according to a predefined standardized data extraction process (Table S3), explained below in detail, with illustrative examples from Foster et al. 1997 in the supplemental materials (Figures S1–S5). Results are stored and available for download in Excel format (https://hawcproject.org/assessment/812/).

First, studies were identified by citation (short and full), and type(s) of study data were indicated (i.e., "Animal bioassay," hereafter referred to as *in vivo* and "*in vitro*"). Studies which contained both *in vivo* and *in vitro* data were indicated as such. In this initial stage of study preparation, general study data (i.e., conflicts of interest, funding source, author correspondence details) were extracted (Figure S1).

Studies with *in vivo* data comprised at least one animal "experiment" (Figure S2). Data extracted within each experiment included study name, study type (acute, short term, subchronic, chronic, mechanistic, reproductive, developmental, etc.), chemical identifiers (name, CAS, source), chemical purity, vehicle used, and details of animal husbandry and diet and compliance with any guidelines for methods (Figure S3). Each experiment consisted of at least one "animal group." Data extracted within each animal group included animal species, strain, and sex, source of animals (laboratory or breeding details), life stage exposed and life stage assessed, observation duration, siblings (if animal groups were related), and any additional description of the animal group (Figure S4). A dosing regimen was also extracted for each animal group, including route of exposure, exposure duration, number of dose groups, control information (positive and/or negative controls), and any other information about dosing methodology (e.g., dose units, dose groups, details of dosing regimen). Dose–response information for end points was also extracted if such data were provided. Each animal group could have multiple end points, characterized by relevant biological system (i.e., reproductive), organ/tissue (i.e., endometrium), effect/effect subtype, diagnostic method, observation time, data set type (e.g., continuous, dichotomous, percent difference), variance type (standard error or standard deviation if reported or relevant), response units, data location in the literature, and other notes on the end point methodology and results (e.g., response direction that would be considered adverse, points of departure, monotonicity, statistical tests, trend tests, etc.) (Figure S4). A dose–response visualization was automatically generated for end points with dose–response data (Figure S5).

A similar data extraction process was performed for *in vitro* studies. First, cell type data was extracted, including species, strain, sex, and tissue of origin (typically female humans), as well as culture type (i.e., primary culture, immortalized cell line, transfected cell line, etc.) and source of cell cultures. Such data were extracted for each relevant cell type used in each study if more than one cell population were used. Chemical exposure data were then extracted, including chemical name, CAS number, source, purity (and purity confirmation details if available), and dilution storage notes. Again, such data were extracted for each unique chemical exposure for each study. Following this initial data extraction, an "experiment" for each cell type was created. If there were multiple cell types in an *in vitro* study, a separate experiment was created for each one. In each *in vitro* experiment, data extracted included dosing information (dosing regimen, duration, units), serum information (percent serum, serum type, and/or description), and control information (positive, negative, and/or vehicle controls). As with *in vivo* experiments, *in vitro* experiments with multiple outcomes were characterized by chemical exposure, assay type, outcome effect, data location in the literature, data set type (e.g., continuous, dichotomous, percent difference), variance type (standard error or standard deviation if reported or relevant), response units, observation time, points of departure, monotonicity, statistical tests, trend tests, and any other notes on the end point of the results. Dose–response data for end points were also extracted when such data were provided. Dose–response visualizations were similarly automatically generated for such end points. Corresponding authors were contacted by email for data unavailable in the published articles and for clarification of methods and risk of bias questions, and authors were provided 2 wk for response.

Synthesis of Results

End points were grouped into primary or secondary end points, as previously explained in the published protocol (Matta et al. 2019). In brief, four primary end points were measured (two *in vivo* and two *in vitro*). For *in vivo* studies, primary end points aimed to be the corollary to endometriosis in humans thus included *a*) the spontaneous onset of endometriosis and *b*) the growth or proliferation of induced endometriotic lesions. For *in vitro* studies, primary end points included *c*) cell migration/invasion and *d*) cell viability/proliferation. Secondary end points included gene expression or protein levels within the signaling pathways regulating the primary end points, markers of disrupted steroidogenic pathways, inflammatory biomarkers, such as cytokines [i.e., interleukins (IL)], or markers of extracellular matrix remodeling [i.e., matrix metalloproteinases (MPPs)]. End point results were summarized in data pivot figures displaying the significance and direction of the effects across exposure doses and studies. The heterogeneity of included studies precluded a quantitative meta-analysis.

Risk of Bias Assessment

The NTP/OHAT RoB Rating Tool was specifically adapted to the research question in the HAWC interface (NTP/OHAT 2015b) and tailored to *in vitro* studies as previously reported (NTP 2016). Briefly, the RoB tool consists of a set of questions tailored to each experimental stream of evidence, addressing six main bias domains listed below (i.e., selection bias, performance bias, attrition bias, detection bias, selective reporting bias, and other) (Table 2).

Questions received one of five possible ratings: "Definitely Low Risk of Bias," "Probably Low Risk of Bias," "Probably High Risk of Bias," "Definitely High Risk of Bias," or "Not Reported," based on prespecified criteria (Supplemental Materials, Section 3). The rating was determined by two independent assessors (K.M. and G.C.S.) and then finalized by discussion and consensus, with

Table 2. Risk of bias analysis: domains of bias and questions.

Domain of bias	Risk of bias question
Selection bias	1. Was administered dose or exposure level adequately randomized?
Selection bias	2. Was allocation to study groups adequately concealed?
Performance bias	3. Were experimental conditions identical across study groups?*
Performance bias	4. Were the research personnel blinded to the study group during the study?
Attrition bias	5. Were outcome data incomplete due to attrition or exclusion from analysis?
Detection bias	6. Can we be confident in the exposure characterization?*
Detection bias	7. Can we be confident in the outcome assessment?*
Selective reporting bias	8. Were all measured outcomes reported?
Other	9. Were there any other potential threats to internal validity?

Note: Key elements considered for the tiered classification are marked by an asterisk (*).

consultation by an additional member of the review team or technical advisors as needed. In the event that additional information was needed to make a rating determination, authors were contacted with questions specific to the RoB question and provided 2 wk to respond. Each RoB rating was justified based on the established criteria and the study text, and is stored and available in HAWC. Based on these ratings, individual studies were ranked on a three-tier scale of bias allowing the classification of specific bodies of evidence in "not serious," "serious," or "very serious" RoB to support decision-making for confidence rating (NTP/OHAT 2015a). More details of the three-tier scale can be found in the next section or in the previously published protocol.

Rating the Level of Evidence

Following the NTP/OHAT framework, we analyzed different domains affecting the confidence level for each primary end point–related body of evidence. Each stream of data was considered a body of evidence, and an assessment was performed for each primary end point to determine a confidence rating reflecting the confidence with which the study findings accurately reflect a true association between exposure to OCCs and the primary end points. The process is summarized in Table S4 and the NTP/OHAT systematic review handbook, based on GRADE guidelines (NTP/OHAT 2015a).

Briefly, each body of evidence was given an initial confidence rating, which was subsequently downgraded or upgraded according to factors that decrease or increase confidence in the results (NTP/OHAT 2015a). The initial confidence rating of "high" was determined by the presence of all four main features determined by the study design for both *in vivo* and *in vitro* evidence (Table S5). This high initial confidence rating was then either downgraded or upgraded, depending on the presence or absence of certain cross-studies flaws (i.e., risk of bias, inconsistency, indirectness, imprecision, publication bias) or strengths (i.e., consistency, large magnitude of effect, dose response), respectively.

Factors decreasing confidence.

1. RoB: As previously mentioned, the NTP/OHAT's RoB-tiered approach considers three key elements of higher relevance to establish the classification criteria for each individual study [marked in Table 2 by an asterisk (*)]. Studies were subsequently categorized into three possible tiers depending on their responses to these key elements: Tier 1: Study must be rated as "Definitely Low" or "Probably Low" RoB for key elements AND have most other applicable items answered "Definitely Low" or "Probably Low"; Tier 2: Study meets criteria for neither Tiers 1 nor 3; Tier 3: Study must be rated as "Definitely High" or "Probably High" RoB for key elements AND have most other applicable items answered "Definitely High" or "Probably High." "Not Reported" was counted as "Probably High." Downgrading for RoB reflects the entire body of studies; therefore, the decision to downgrade was applied conservatively and reserved for cases with substantial RoB across most of the studies composing the body of evidence.
2. Unexplained inconsistency: Studies were considered for downgrading when there was inconsistency in results that were not explained by study design features, such as differences in cell model/animal species, timing or route of exposure, or health outcome assessment.
3. Indirectness/applicability: The following points were used to assess the directness in the present study:
   a. Differences in population (applicability) and relevance of the animal model to outcome of concern.
      i. *In vivo* studies: Studies conducted in mammalian model systems were assumed relevant for humans (i.e., not downgraded) unless compelling evidence to the contrary was identified during the course of the evaluation (e.g., a biological system not present in humans).
      ii. *In vitro* studies: Cell models were evaluated on the basis of the biological relevance in humans (human primary cell cultures or human immortalized cell lines).
   b. Differences in outcome measures or directness of the end points to the primary health outcome(s). For example, onset of endometriosis would be a direct end point, whereas development of "endometriosis-like" phenotypes is less direct.
   c. Dose levels and route of administration in *in vivo* studies: External dose comparisons were used to reach confidence rating conclusions, because internal dosimetry in animal models can vary based on route of administration, species, age, diet, and other cofactors. The most commonly used routes of administration (i.e., oral, dermal, inhalation, subcutaneous injections) were considered direct for the purposes of establishing confidence ratings.

   The applicability of specific health outcomes or biological processes in animal models is outlined in the PECO inclusion/exclusion criteria, with the most accepted relevant/interpretable outcomes considered "primary," and less direct measures, biomarkers of effect, or upstream measures of health outcome considered "secondary."
4. Imprecision: Imprecision is typically assessed with confidence intervals for meta-analyses, but because a meta-analysis was not performed due to the heterogeneity of outcome measurements, the overall effects of the studies were considered for imprecision. Studies with high variability of effect estimates were at risk of imprecision bias.
5. Publication bias: Studies were considered for downgrading for publication bias when the study was uniformly small, especially when sponsored by industries, nongovernment organizations, or authors with conflicts of interest. Abstracts or other types of gray literature that do not appear as full-length articles within a reasonable time frame (3–4 y) may be another indication of publication bias; thus such literature has been excluded.

Factors increasing confidence.

1. Magnitude of effect: A large magnitude of effect was considered to upgrade the confidence.
2. Dose–response: Confidence was upgraded for dose–response if there was sufficient evidence of monotonic dose–response/gradient.
3. Consistency: Consistency across animal studies, dissimilar populations, and study types were potential reasons to upgrade the confidence:
   a. Across animal studies: consistent results reported in multiple experimental animal models or species. Finding the same direction of change in the same outcome in more than two species would constitute sufficient evidence that a causal relationship has been established, by standards set by the International Agency for Research on Cancer. Though the health effect studied here is not cancer, the same principle can apply.
   b. Across dissimilar populations: consistent results reported across populations that differ in factors such as time, location, and/or exposure.
   c. Across study types: consistent results reported from studies with different design features (e.g., between chronic and multigenerational animal studies).

Rating the Level of Evidence

Subsequently, the confidence rating was translated to the level of evidence considering the presence or absence of the health effect and the direction or nature of the effect (Table S6). Level of evidence was established for each of four primary outcomes separately.

Evidence Integration and Hazard Identification

Based on the NTP/OHAT Hazard Identification Scheme (Figure S6) reported by Rooney et al. (2014), the level-of-evidence conclusion for human data can be considered together with nonhuman animal data to reach a hazard identification conclusion. At the first stage of integration, the level of evidence (i.e., "high," "moderate," "low") from human and nonhuman animal studies is considered in order to establish a preliminary classification (i.e., "known," "suspected," "presumed," "not classifiable"). In a second step, the impact of *in vitro* data can be used to bolster or downgrade this preliminary classification. We thus considered the epidemiological evidence meta-analyzed and evaluated in our previously published systematic review following the same guiding framework (Cano-Sancho et al. 2019) with the results of this review to reach a hazard identification conclusion.

The epidemiological evidence was grouped by main OCC families with enough studies to conduct a pooled analysis, including PCDD, PCDFs, PCBs, or OCPs. The body of evidence for PCDD/PCDFs consisted of 10 epidemiological studies, mainly reporting associations between TCDD, total dioxin toxic equivalents (TEQ), or TEQ for the group PCDD/PCDFs. The pooled effect size resulted in an odds ratio (95% confidence interval) of 1.65 (1.14, 2.39) with substantial heterogeneity ($I^2 = 72\%$). The sources of heterogeneity were identified by stratified analysis, including the biological matrices or analytical methods, preventing the downgrading due to unexplained inconsistency. The confidence was rated as moderate and translated into a "moderate" level of evidence of health effect.

Results

Through the search and selection process, we identified a total of 812 unique articles, of which 716 were excluded through Phase I screening based on title and abstract (Figure 1). The full texts of 96 articles were then examined during Phase II review. A total of 39 of these articles met the eligibility criteria (Table 1), 16 of which contained *in vivo* data and 24 *in vitro* data, among which one

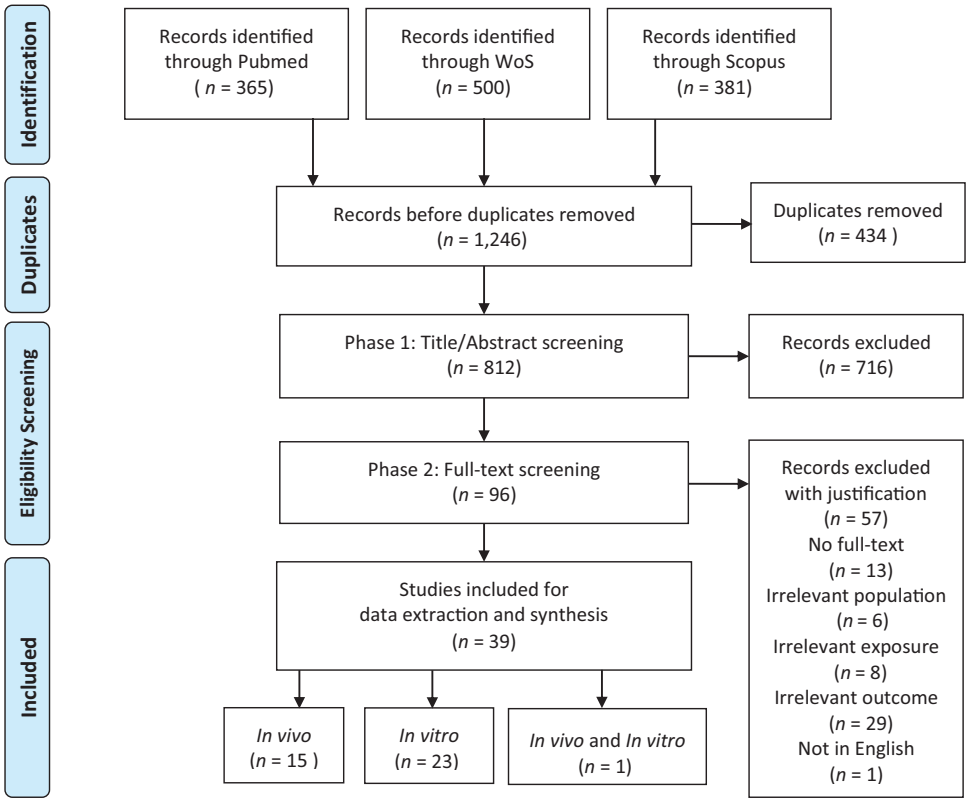


Figure 1. PRISMA flowchart displaying the results from the literature search and screening.

Table 3. Summary table of selected *in vivo* studies.

Reference	Species	Chemical	Study type	Size	Dosing duration/frequency	Route	Doses	Units	Reported end point	Consolidated end point
Arnold et al. 1996	Rhesus monkey	Aroclor 1254	Chr	16	10 y	cap	0/5/20/40/80	g/kg body weight	Incidence	Onset
Rier et al. 1993	Rhesus monkey	TCDD	Chr	8 (24)	4 y	diet	0/5/25	ppt	Incidence	Onset
Yang et al. 2000	Cynomolgus monkey	TCDD	Chr	5-6	1 y (1 capsule 5 d/wk)	cap	0/1/5/25	ng/kg/d	Diameter	Lesion growth
Bruner-Tran et al. 1999	Nude mouse	TCDD	ST	10	Single dose	subq	0/1	nM	Amount	Onset
Nayyar et al. 2007	C57BL/6 mouse	TCDD	Dev	5-6	6 dose combinations Utero/prepubertal/pubertal	gav	0/10	µg/kg	Like phenotypes	Onset
Cummings et al. 1999	B6C3F1 mouse	TCDD	Dev	3 (12)	2 doses (prenatal postnatal)	gav	0-0/0-3/3-0/3-3/3-10	ug/kg	Diameter	Lesion growth
	SD rat	TCDD	Dev	3 (12)	2 doses (prenatal postnatal)	gav	0-0/0-3/3-0/3-3/3-10	mg/kg	Diameter	Lesion growth
Cummings et al. 1996	B6C3F1 mouse	TCDD	SChr	8 (32)	96 d (1 dose/3 wks)	gav	0/3/10	ug/kg	Diameter	Lesion growth
	SD rat	TCDD	SChr	8 (32)	0, 3, 6, 9, 12 wk	gav	0/3/10	µg/kg	Diameter	Lesion growth
Foster et al. 1997	B6C3F1 mouse	TCDD	SChr	8, 5	30 d (daily dose)	subq	0/100	ng/kg/d	Diameter	Lesion growth
		4-CDE	SChr	9, 6	30 d (daily dose)	subq	0/150	mg/kg/d	Diameter	Lesion growth
Huang et al. 2017	B6C3F1 mouse	PCB 153	ST	12	Single dose (10 or 20 d)	gav	0/5/50	mg/kg	Amount/Weight	Lesion growth
		PCB 126	ST	12	Single dose (10 or 20 d)	gav	0/0.03/0.3	mg/kg	Amount/Weight	Lesion growth
Johnson et al. 1997	B6C3F1 mouse	TCDD	Chr	10	15 wk (5 doses/3 wk)	gav	0/1/3/10	µg/kg body weight	Diameter/Weight	Lesion growth
		PCB 153	Chr	10	15 wk (5 doses/3 wk)	gav	0/3/30	mg/kg body weight	Diameter/Weight	Lesion growth
		PCB 126	Chr	10	15 wk (5 doses/3 wk)	gav	0/100/300/1000	µg/kg body weight	Diameter/Weight	Lesion growth
		1,3,6,8-TCDD	Chr	10	15 wk (5 doses/3 wk)	gav	0/2/20	mg/kg body weight	Diameter/Weight	Lesion growth
		4-PeCDF	Chr	10	15 wk (5 doses/3 wk)	gav	0/10/30/100	µg/kg body weight	Diameter/Weight	Lesion growth
Khan et al. 2018	C57BL/6 mouse	TCDD	SChr	10	9 wk (3 doses/3 wk)	gav	0/3	µg/kg body weight	Diameter	Lesion growth
Kitajima et al. 2004	B6C3F1 mouse	TCDD	ST	3	Single dose (4 wk after induction)	subq	0/10	µg/kg body weight	Diameter/gene expression	Lesion growth
Yang and Foster 1997	B6C3F1 mouse	TCDD	ST	5	28 d (daily dose)	subq	0/10/50/100	ng/kg/d	Diameter/Adhesion	Lesion growth
Yang et al. 1997	B6C3F1 mouse	4-CDE	ST	5 (25)	28 d (daily dose)	subq	0/10/75/150	mg/kg/d	Diameter	Lesion growth
Chiappini et al. 2019	SD rat	HCB	SChr	7	30 d (3 doses/wk)	gav	0/1/10/100	mg/kg body weight	Volume /Diameter	Lesion growth
Cummings and Metcalf 1995b	SD rat	MCX	ST	10	21 d (daily dose)	gav	0/250	mg/kg/d	Diameter	Lesion growth

Note: 4-CDE, 4-chlorodiphenyl ether; Chr, chronic; Dev, developmental; cap, oral capsule; gav, oral gavage; ST, short term; SD, Sprague Dawley; SChr, subchronic; subq, subcutaneous injection. Size listed is animals per dose group (total in parentheses when provided). Separate doses are separated by slashes (/), whereas multiple doses are separated by a dash (–).

reported both data streams. The included articles are summarized in Tables 3 and 4.

Study Characteristics

Main characteristics from *in vivo* studies are summarized in Table 3. Four animal models were identified, including nonhuman primates ($n = 3$), nude mice ($n = 1$), autologous mice ($n = 9$), and autologous rats ($n = 4$). Two studies tested both rat and mouse models simultaneously (Cummings et al. 1996, 1999). For primates and mouse models where spontaneous endometriosis or endometriosis-like phenotype could occur, the consolidated "endometriosis onset" end point was generated, grouping both incidence of phenotypes and attachment of injected endometrial tissue. For models in which endometriosis was surgically induced by sewing autologous endometrium to peritoneal tissues or vessels, lesion growth was the primary end point. Presence of endometriosis and size of endometriotic lesions were confirmed histologically, through laparoscopy or necropsy for all studies. A total of 14 OCCs were assessed across all studies (Table S7), but the majority of studies ($n = 27$) assessed TCDD, the prototypical ligand of the AhR; other chemicals assessed by multiple studies were PCB 126 ($n = 5$), PCB 153 ($n = 4$), and DDT isomers ($n = 4$). PCBs 77 and 104; 1,3,6,8-TCDD; 4-PeCDF; and pesticides hexachlorobenzene (HCB), atrazine (ATR), and methoxychlor (MXC) were only assessed by a single study. Studies were either chronic (>90 d, $n = 8$), subchronic (30–90 d, $n = 6$), short term (<30 d, $n = 7$), or developmental ($n = 3$) (Table 3). We did include one review as an exception, because it presented primary data generated by the authors (Bruner-Tran et al. 1999).

Main characteristics from *in vitro* studies are summarized in Table 4. A variety of cell types have been assessed (Table S8), often with multiple cell types in a single study. Cell models included endometrial stromal cells (ESCs) ($n = 16$), ESC cocultures ($n = 10$), endometrial endothelial cells (EEnCs) ($n = 2$), and endometrial explants ($n = 2$), as well as uterine fibroblasts (HUF), granulosa cells, and healthy noncancer endometrial cells. Three immortalized cell lines were included: ESCs immortalized with temperature-sensitive SV40 T antigen (ESC-SV40T), human telomerase reverse transcriptase (hTERT) EECs, and T-HESC, a uterine fibroblast immortalized with hTERT. All other cell models were primary cultures from human biopsies. Primary *in vitro* end points included cell migration/invasion as well as cell viability/proliferation.

Associations between Exposure to OCCs and Primary and Secondary Outcomes

OCCs and endometriosis onset. Four *in vivo* studies measured the development of spontaneous endometriosis or endometriosis-like phenotypes, including two primate studies (Arnold et al. 1996; Rier et al. 1993) and two rodent studies (Bruner-Tran et al. 1999; Nayyar et al. 2007). One study with rhesus monkeys assessed exposure to Aroclor 1254 and the other studies to TCDD, with different animal models, exposure regimens, and outcome definition. The study on Aroclor 1254 did not find a significant relationship between incidence of endometriosis and dose treatment, regardless of concentrations (up to 80 µg/kg body weight per day) or follow-up duration. The high incidence in controls (37%) precluded the role of PCB exposure in increasing either incidence of endometriosis or the severity of the lesions [25% (16/64)] of treated monkeys (Arnold et al. 1996). The three studies on TCDD exhibited positive associations at different doses and using different models. Rier et al. (1993) noted a statistically significant dose-dependent increase in incidence and severity of endometriosis in a chronic study (4 y). Within the

Table 4. Summary table of selected *in vitro* studies.

Reference	Tissue/cell type	*n*	Chemical	Duration	Dose range	Unit	Assay	Reported end point	Consolidated end point
Bofinger et al. 2001	EExCU (endo and no endo)	9-11	TCDD	24 h	0.001–10	nM	Northern blot (mRNA) EROD assay	CYP1A1/B1 mRNA EROD activity	Steroidogenesis$^{(s)}$
Bredhult et al. 2007	EEnC	6	op′-DDT	24 h	1–100	μM	ICC PCNA, BrdU Assay	Viability/proliferation	Viability/proliferation
	EEnC	6	TCDD	24 h	0.1–100	nM	ICC PCNA, BrdU Assay	Viability/proliferation	Viability/proliferation
	EEnC	6	PCB 77, 126	24 h	0.1–10	μM	ICC PCNA, BrdU Assay	Viability/proliferation	Viability/proliferation
Bredhult et al. 2008	EEnC	10	op′-DDT	24 h	50	μM	BrdU Assay/qRT-PCR	Proliferation	Proliferation
Chang et al. 2017	ESC/monocyte*	6	TCDD	48 h	0.1–5	nM	Flow cytometry	IL-10	Inflammation$^{(s)}$
Chiappini et al. 2016	HUF, ESC (EU (endo and no endo)/EN), T-HESC	3	HCB	24 h	0.005–5	μM	MTT/WB	Viability MMP (−2, −9)	Viability, inflammation$^{\%m}$ steroidogenesis$^{(s)}$, migration/invasion
Holloway et al. 2008	ESC Granulosa Cells	10/5	Atrazine	24 h	0.001–100	μM	TWE	Aromatase activity	Steroidogenesis$^{(s)}$
Holloway et al. 2005	ESC	9	p,p′-DDE	24 h	1–10,000	ng/mL	TWE	Aromatase activity	Steroidogenesis$^{(s)}$
Hu et al. 2018	EU ESC (endo and no endo)	9	PCB 104	12 h–72 h	2–10	μM	rtPCR/ELISA/CCK8/Crystal Violet	Gene expression/protein levels/ proliferation/migration	Viability/proliferation, migration, inflammation$^{(s)}$
Huang et al. 2017	ESCs (EU/EN)	3	PCB 126, 153	48 h	0.3–60	μM	MTT/ELISA/WB	Cell viability IL-6, IL-8 s	Viability, inflammation
Igarashi et al. 2005	ESC-EEC*	3	TCDD (E)	48–72 h	0.1–20	nM	RT-PCR/ WB	PR-B/PR-A ratio MMP (−3, −7)	Steroidogenesis$^{(s)}$, inflammation
Kalinina et al. 2018	Normal ESC	5	DDT	24 h	1–10	μM	RT-PCR	microRNA-190a/b	Migration/invasion
Li et al. 2011	4 ESC (Normal, CD82-, EC, EU)	6	TCDD (E)	48 h	10	nM	MTWA/ELISA	Invasion, Chemokine CCR2	Invasion, inflammation$^{(s)}$
Pitt et al. 2001	EExCU	11-13	TCDD (EP)	24–72 h	0.001–10	nM	qRT-PCR	AhR mRNA	Steroidogenesis$^{(s)}$
Resuehr et al. 2012	ESC	5	TCDD	48 h	10	nM	qRT-PCR	CB1-R, PR-B	Inflammation$^{(s)}$/steroidogenesis$^{(s)}$
Shi et al. 2006	ESC (EU/EN) ESC-PMC *	3	TCDD (E)	48 h	1	nM	ELISA/IMS DNA	IL-8 /CXCR1	Inflammation$^{(s)}$
Shi et al. 2007	ESC (EU/EN)	3	TCDD (E)	48 h	0.01–10	nM	Flow cytometry	CCR8	Inflammation$^{(s)}$
Van den Brand et al. 2019	ESC-EEC*	3	TCDD (EP)	48 h	1–500	pM	TWE	AhR/CYP1A1	Steroidogenesis$^{(s)}$
Wang 2015	ESC-U937 macrophage *	3	TCDD (E)	48 h	0.01–10	nM	ELISA/flow cytometry	IL-10, IL-12/CD86	Inflammation$^{(s)}$
Wang et al. 2010b	ESC(EC/EU), ESC-U937* ESC-HMPC-U937 *	3	TCDD (E)	48 h	1	nM	Western blot, Flow cytometry, ICC	TECK/CCR9 MMP (−2, −9)	Migration/invasion, inflammation$^{(s)}$
Wang et al. 2010a	ESC (EC/EU)-U937* ESC-HMPC-U937 *	3	TCDD (E)	48 h	1	nM	Chemotaxis cell migration assay WB	Macrophage migration Chemokine CCR1 Expression	Migration, inflammation$^{(s)}$
Willing et al. 2011	hTERT-EEC	3	TCDD (E)	24 h	1–10	nM	LRGA/WB/proteomics Alamar Blue	AhR /CYP1A1, migration Proliferation, ARNT	Steroidogenesis$^{(s)}$, migration, proliferation
	hTERT-EEC	3	PCB 126, 153	24 h	100	ng/mL	LRGA/WB/proteomics Alamar blue	AhR CYP1A1, migration Cell proliferation, ARNT	Steroidogenesis$^{(s)}$, migration, proliferation
Yang 1999	ESC-SV40T	3	TCDD	24 h	0.1–100	nM	qRT-PCR	AhR /IL-1B /PAI-2 mRNA	Steroidogenesis$^{(s)}$, inflammation$^{(s)}$ migration/invasion
Yu et al. 2008	ESCs (EC/EU)-ESC/HMPC-U937*	3	TCDD (E)	48 h	1	nM	Matrigel-based transwell ELISA, western blot	Cell invasion, MIP-1α secretion, RANTES, MMPs	Invasion, inflammation$^{(s)}$
Zhao et al. 2002	ESCs (normal/EN)	3	TCDD	24 h	10	nM	PCR, ELISA, luciferase reporter gene	RANTES (mRNA, Protein secretion, gene expression)	Inflammation$^{(s)}$

Note: "Endo and no endo" is used to describe tissue/cell cultures drawn from women with and without endometriosis. Co-cultures are marked by an asterisk (*); *n* refers to number of replicates or explants per dose group or cell type. Cultures concomitantly treated with estrogen and/or progesterone as well as TCDD are marked by E and/or P. Dose ranges exclude control group (0). Secondary outcomes are identified with "$^{(s)}$" in table. DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; E, estrogen; EC, ectopic; EEnCs, human endometrial endothelial cells; ELISA, enzyme-linked immunosorbent assay; EExCU), endometrial explant culture; EN, endometriotic; EROD, 7-ethoxyresorufin O-deethylase; EECs, endometrial epithelial cell; ESCs, endometrial stromal cells; ESC-SV40T, ESCs immortalized with temperature-sensitive SV40 T antigen; EU, eutopic; hTERT-EEC, human telomerase immortalized reverse transcriptase endometrial epithelial cell; HUF, human uterine fibroblast; ICC, immunocytochemistry; IMS, immunostaining; LRGA, luciferase reporter gene assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; MTWA, Matrigel-based transwell western assay; P, progesterone; PCB, polychlorinatedbiphenyl; PCR, polymerase chain reaction; PMC, peritoneal mesothelial cell; qRT-PCR, quantitative real-time polymerase chain reaction; TDCC, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; T-HESC, telomerase-immortalized human endometrial stromal cells; TWE, tritiated water-release; WB, Western-blot.

control group, 2 out of 6 (33%) monkeys developed endometriosis, whereas at the 5 and 25 ppt dose groups, incidence was 5 out of 7 (71%) and 6 out of 7 (86%), respectively (Rier et al. 1993). In a mouse model, the incidence of endometriotic-like lesions was measured 10 d after the mice were injected intraperitoneally with proliferative-phase endometrial tissues treated for 24 h with TCDD (1 nM) combined with progesterone (P) or estradiol (E2) (Bruner-Tran et al. 1999). The combined E2 + P + TCDD treatment ($n = 8$) led to 42 total lesions, whereas mice with the E2 treatment alone ($n = 8$) or combined with P developed 20 total lesions or 0, respectively. In a developmental study, Nayyar et al. (2007) examined the presence of uterine phenotypes in mice, noting similarities in the phenotypes exhibited in the TCDD-treated animals to those found in humans with endometriosis. C57BL/6 mice were exposed to 10 μg TCDD/kg body weight at different combinations of critical developmental life stages (six treatment groups, singly or in combination of: in utero/lactation, and/or prepubertal, and/or pubertal). Authors reported the similarities through immunohistochemical imaging but did not provide statistical analyses.

OCCs and endometriotic lesion growth. A total of 12 *in vivo* studies measured the change in endometriotic lesion size in diameter, volume, and/or weight following the exposure to TCDD (Figure 2A) and other OCCs (Figure 2B). Most studies were performed on rodent models ($n = 9$ mice, $n = 4$ rats), and one on cynomolgus monkeys. TCDD was analyzed in 8 out of 12 studies, with variable results, appearing mostly positively associated at the highest doses and null or negatively associated with lesion diameter and/or weight at the lowest doses (Figure 2A). The three studies which report negative associations had provided animals with a high dose pretreatment of E2 (24 ng/kg/d) or estrone (E1) (20 μg/kg/d) (Foster et al. 1997; Kitajima et al. 2004; Yang and Foster 1997). E2 did significantly increase epithelial height, stromal thickness, and proliferative activity of the endometriotic lesions, whereas coadministered TCDD reduced these effects (Kitajima et al. 2004), supporting the antiestrogenic effects of TCDD. Other dioxin-like chemicals were also tested, including 4-PeCDF, which significantly increased lesion weight at the highest dose (100 μg/kg body weight) (Johnson et al. 1997) and PCB 126 (dioxin toxic equivalency factor of 0.1), which led to increased lesion diameter and weight in two studies (Huang et al. 2017; Johnson et al. 1997). Two noncoplanar chemicals were also analyzed (1,3,6,8-TCDD and PCB 153), neither of which significantly affected lesion size (Johnson et al. 1997). Proestrogenic 4-chlorodiphenyl ether (4-CDE) was found to increase lesion diameter at all tested doses but only significantly at the highest dose (150 mg/kg/d); this increase was not as much in comparison with that of the E2-treated positive control (Foster et al. 1997; Yang et al. 1997). MXC (250 mg/kg/d, for 21 d by oral gavage) significantly increased lesion diameter in comparison with the vehicle control (Cummings and Metcalf 1995b). Lesion diameter increased dose dependently in rats treated with HCB through oral gavage (1, 10, and 100 mg/kg body weight), 3 times a week for 30 d (Chiappini et al. 2019). Overall, results support the hypothesis that exposure to TCDD and dioxin-like OCCs contribute to an increase in the growth of endometriotic lesions at the highest concentrations in comparison with negative or vehicle-treated controls, whereas exposure to noncoplanar PCBs and other OCCs did not have such a consistent effect, suggesting the involvement of the AhR signaling pathway.

OCCs and endometrial cell viability/proliferation. Cell viability was measured for six different chemical assays from three *in vitro* studies, and proliferation was measured for six chemicals in eight studies using five cellular models (EEnC, ESC, hTERT-EEC, HUF, and T-HESC). The overall evidence on viability and proliferation was inconclusive (Figure 3A,B). One study reported a significant increase of proliferation of endometrial cells dosed with PCB 126, PCB 153, and TCDD (Willing et al. 2011), whereas the rest reported mostly null results or significant decreases of viability or proliferation, especially at high doses. This discrepancy may likely be due to a trait in the immortalized hTERT-EEC cell line, which is often used for cancer studies; authors observed that TCDD has no antiestrogenic effects in hTERT-EEC, becayse it failed to inhibit estrogen-induced ER-alpha down-regulation in the presence of estrogen (Willing et al. 2011).

OCCs and* in vitro *cell migration/invasion. Six studies measured changes in cell migration and invasion for endometrial cells and co-cultures dosed with TCDD or PCBs (Figure 4A,B). Molecular markers supporting cell migration/invasion are reported in Figure 4B. Results across models and doses consistently showed increase in cell motility. In two studies, TCDD alone was not found to increase invasiveness, but the combination of TCDD with E2 did lead to a significant increase (Wang et al. 2010a; Yu et al. 2008). Three studies observed that the combination of E2 with TCDD had a synergistic effect on increased cell invasion (Li et al. 2011; Wang et al. 2010b; Yu et al. 2008). Additionally, ESCs in a co-culture with HPMC-U937 cells tended to have higher motility than ESCs cultured alone (Wang et al. 2010a; Yu et al. 2008). Overall, OCC exposures (especially in the presence of estrogens) was found to contribute to increased migration and invasion of endometrial cells.

OCCs and extracellular matrix remodeling. MMPs are a group of enzymes involved in the restructuring of endometrial tissue during the proliferative phase and are responsible for regulation of cell cycle and endometrial tissue remodeling (Nagase et al. 2006). Elevated MMP activities have been associated with extracellular matrix degradation and angiogenesis (Nanda et al. 2020) and play a critical role in the proliferation and invasion of endometriotic cells (Samartzis et al. 2019; Weigel et al. 2012). Six studies reported associations between OCC exposure and MMP activity and expression (Figure 5), exhibiting consistent positive associations for TCDD, PCBs, and HCB with varying degrees of significance. The exposure of eutopic ESCs to PCBs 104, 126, and 153 showed significant increases in MMP-2, MMP-3, MMP-9, and MMP-10 activity and expression. HCB increased MMP-2 and MMP-9 activities in HUF, T-HESC, and ESCs. MMP-9 levels were consistently elevated in all models, whereas MMP-2 was significantly elevated only in ESCs (Chiappini et al. 2016). One study reported a synergistic interaction when ESCs were dosed with a combination of 1 nM TCDD and E2, significantly increasing MMP-2 and MMP-9 activities, whereas TCDD alone had no significant effect (Yu et al. 2008).

OCCs and markers of inflammation. Markers of inflammation and immune dysfunction, including cytokine activity, were extensively evaluated in *in vitro* studies, representing the most diverse and substantiated body of indirect evidence of associations with endometriosis (Figure 6). The evaluation included studies mostly focused on the effects of TCDD (alone or combined with E2); few studies tested the effects of other OCCs on inflammatory cytokines, including o,p′-DDT, HCB, PCB 126, and PCB 153 (Bredhult et al. 2008; Chiappini et al. 2016; Huang et al. 2017). The results globally support a perturbation of immune function and inflammation of endometrial cells and co-cultures with macrophages, for the different OCCs.

Prostaglandin E2 (PGE2) is an important mediator of inflammatory responses and is derived from arachidonic acid metabolized by cyclooxygenase-2 (COX-2) enzymes (Herr 2012; Park et al. 2006). In T-HESCs, HCB stimulated the c-Src kinase-induced COX-2 expression and activity, subsequently increasing the production and secretion of PGE2 and the expression of its

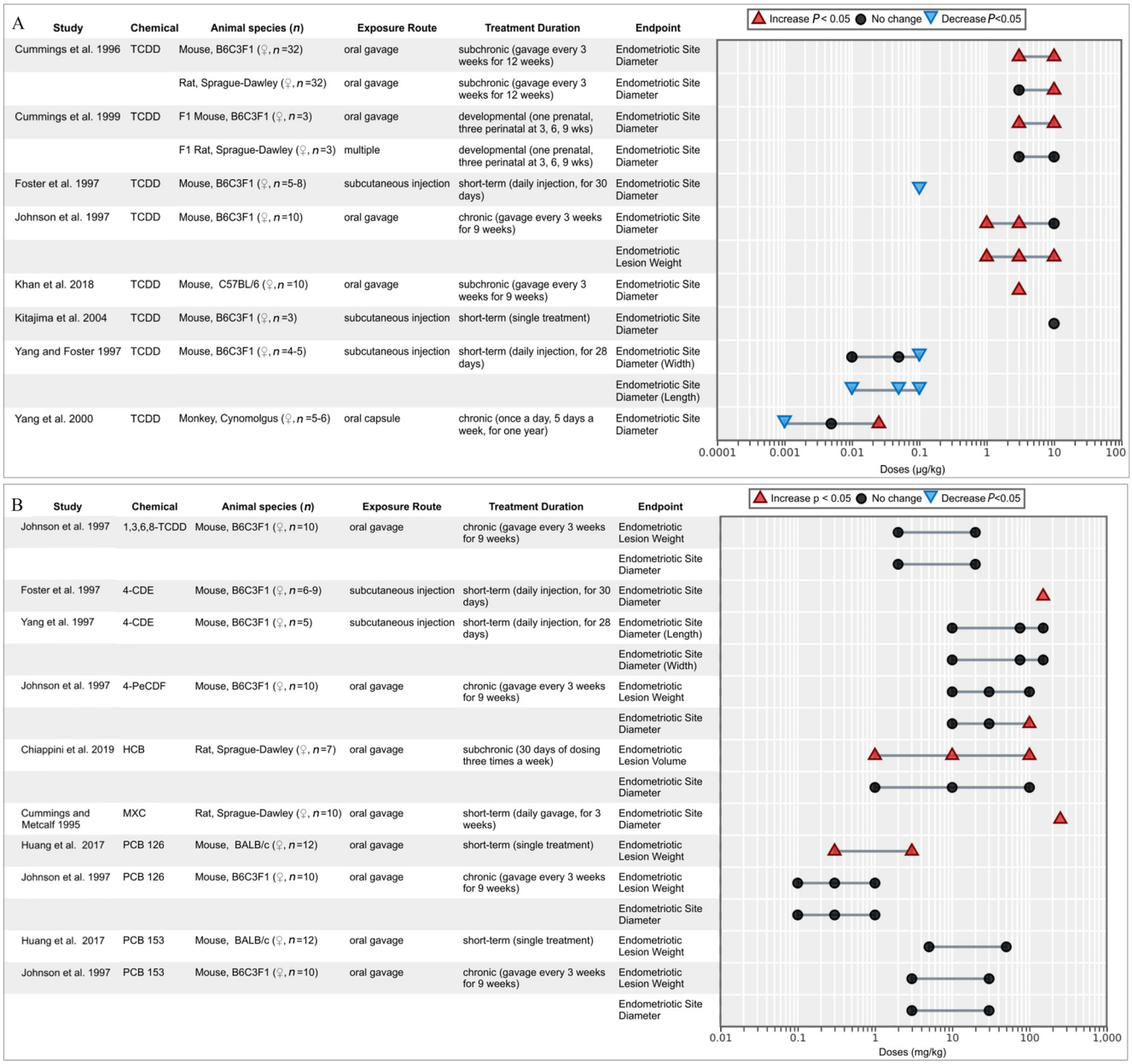


Figure 2. Associations between endometriotic lesion growth (measured in diameter, volume, and weight) reported from *in vivo* studies for TCDD (2A) and other organochlorine chemicals (2B). Lesion growth was measured in diameter, volume, and weight.

mediator G-protein coupled receptor EP4 (Chiappini et al. 2016). Elevated COX-2 and PGE2 expression promoted the proliferation and invasion of ESCs (Takenaka et al. 2010). PGE2 was found to bind to EP4 and trigger c-Src kinase phosphorylation, MMP activation, and the migration and invasion of human immortalized endometriotic epithelial and stromal cells (Lee et al. 2011). Huang et al. (2017) examined the effects of nondioxin-like PCB 153 and dioxin-like PCB 126 on ESCs and found that exposure to PCB 126 decreased anti-inflammatory lipid mediator lipoxin A4 (LXA4). Secretion of IL-6, a proinflammatory interleukin, was also found to increase in ESCs in response to both PCBs 126 and 153 (Huang et al. 2017).

Protein secretion of proinflammatory chemokine RANTES (CCL5) was found to significantly increase in ESCs and U937-ESC-HMPC co-cultures in response to the combination of TCDD and E2, but not TCDD alone (Yu et al. 2008). Both RANTES gene expression and protein secretion increased in both ectopic and eutopic ESCs exposed to TCDD (Zhao et al. 2002). CCR8 expression increased in a bell shaped distribution with increasing dose of TCDD, especially in combination with E2, for eutopic and ectopic ESCs, and especially when co-cultured with U937 and HMPC cells (Shi et al. 2007). CCR9 expression increased in response to TCDD in U937-ESC-HMPC co-culture but not U937-ESC co-culture without HMPC, suggesting cell cross talk plays a role (Wang et al. 2010b). The CCL2 chemokine secretion and CCR2 protein levels (receptor expression) was significantly elevated in normal healthy ESCs following TCDD exposure—an increase that was even stronger in CD82 silenced cells (Li et al. 2011). In turn, CCR1 protein translation significantly increased in ESC co-cultures exposed to TCDD and E2 combined, as well as U937-ESC-HMPC triple co-culture treated with TCDD alone, but not U937-ESC double co-culture treated with TCDD alone, again supporting cross talk (Wang et al. 2010b). Secretion of IL-8 and expression of its receptor CXCR1 both increased in ESCs exposed to TCDD (Shi et al.

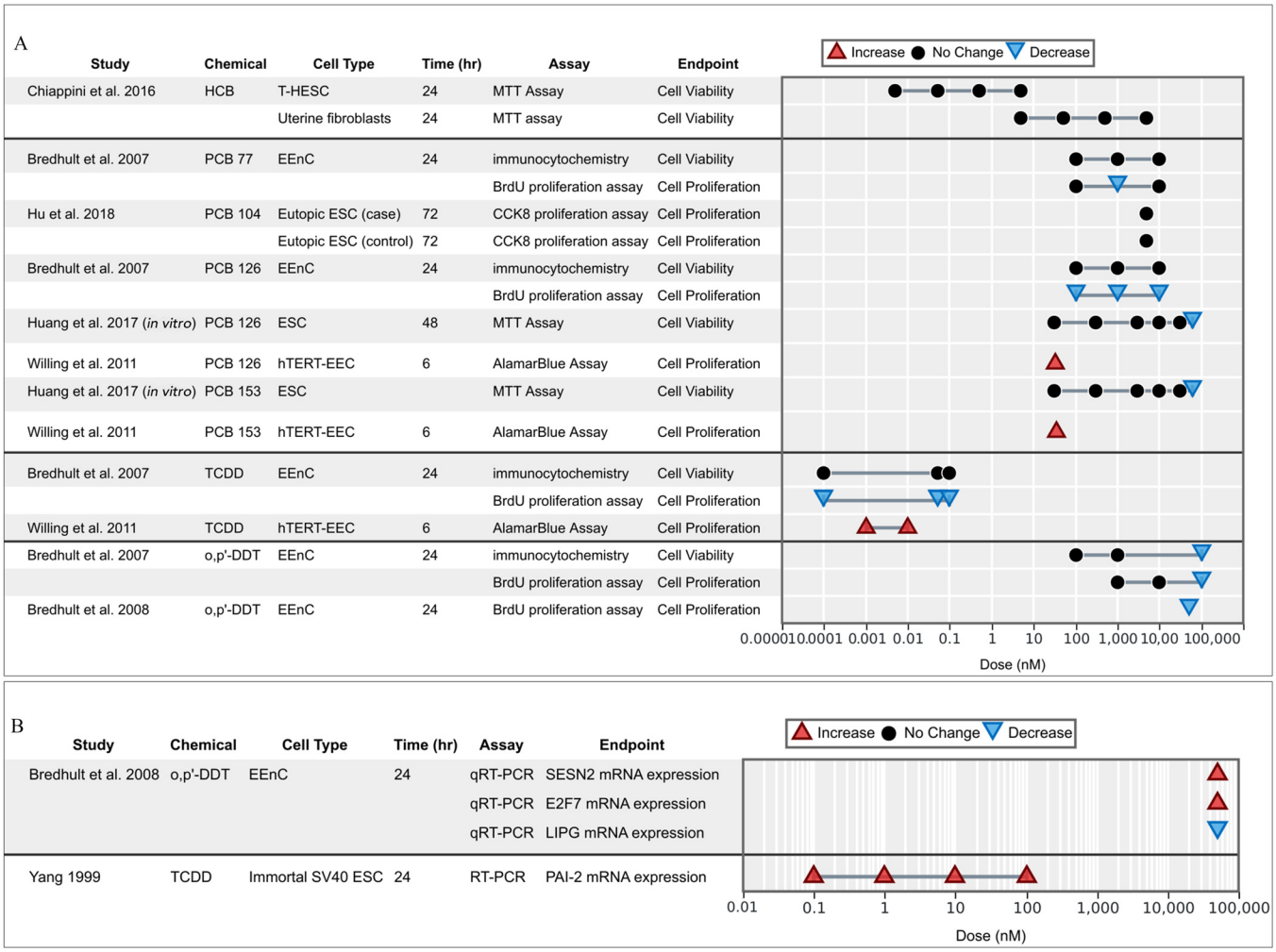


Figure 3. Associations between exposure to organochlorine chemicals and (A) cell viability/proliferation and (B) molecular markers supporting viability/proliferation from *in vitro* studies. Endometrial stromal cells (ESCs) marked with case or control indicate that the cells were derived from women with endometriosis (case) or without (control).

2006). Combined TCDD and E2 treatment inhibited CD86 expression in a dose-dependent manner in ESCs co-cultured with a macrophage cell line, which contributed to an increase in anti-inflammatory cytokine IL-10 (and not IL-12) production, providing evidence for the role of M2 macrophage activation in promoting endometriosis (Wang et al. 2015). IL-27, a member of IL-12 family, was significantly up-regulated in the ESC-monocyte co-culture after exposure to TCDD, further supporting cross talk between macrophages and ESCs (Chang et al. 2017). It is suggested that secretion of IL-27 by macrophages and ESCs induces IL-10 production in Th17 cells; this suggestion is also supported by *in vivo* evidence from the same study in which the IL treatments (50 µg of IL-27, IL-10, and IL-17A) were tested on a nude C57BL/6 endometriosis mouse model, which increased the number and weight of endometriotic lesions (Chang et al. 2017). It is also suggested that the balance between M1 and M2 macrophages may contribute to angiogenesis and implantation of endometrial cells in pelvic endometriosis (Wang et al. 2013). The secretion of macrophage inflammatory protein MIP-1α was found to increase significantly after 48 h of exposure to 1 nM TCDD (Yu et al. 2008). One study hypothesized that increased invasiveness could be due to increased secretion of the chemokine TECK (CCL25), which plays a key role in compartmentalization of the mucosal immune system through recruitment of localized immune cells (Wang et al. 2010b). In this regard, TECK was up-regulated in a ESC–HPMC–U937 co-culture exposed to 48 h of E2 (10 nM) with TCDD (1 nM). This increase was observed in only the three-cell co-culture and not in ESCs tested alone, nor in either of the other cell types tested alone, suggesting cellular cross talks. Both the introduction of ESCs and the combination of E2 and TCDD increased TECK secretion in the endometriosis-associated cells and promoted the invasiveness of ESCs by increasing expression of MMP-2 and MMP-9 (Wang et al. 2010b).

OCCs and P resistance. The disruption of P response is understood to be strongly linked to endometrial diseases (Fu et al. 2017) because the P receptor (PR) serves a protective role in modulating the impacts of estrogens and xenoestrogenic compounds (Yilmaz and Bulun 2019). Especially important is the relative expression of its isoforms PRA and PRB; PRB is often found to be suppressed or completely absent in tissues affected by endometrial diseases (Arnett-Mansfield et al. 2001; Brayman et al. 2006; Shao 2013). Overall, results consistently showed the suppression of the PRB in response to OCCs (Figure 7A). The PRB/PRA ratio was found to be significantly decreased in ESCs exposed to TCDD in all tested doses (Igarashi et al. 2005). TCDD also significantly inhibited overall PRB expression (Resuehr et al. 2012).

Endometrial CB1-R immunoreactivity is dysregulated in women with endometriosis (Resuehr et al. 2012). P was found to

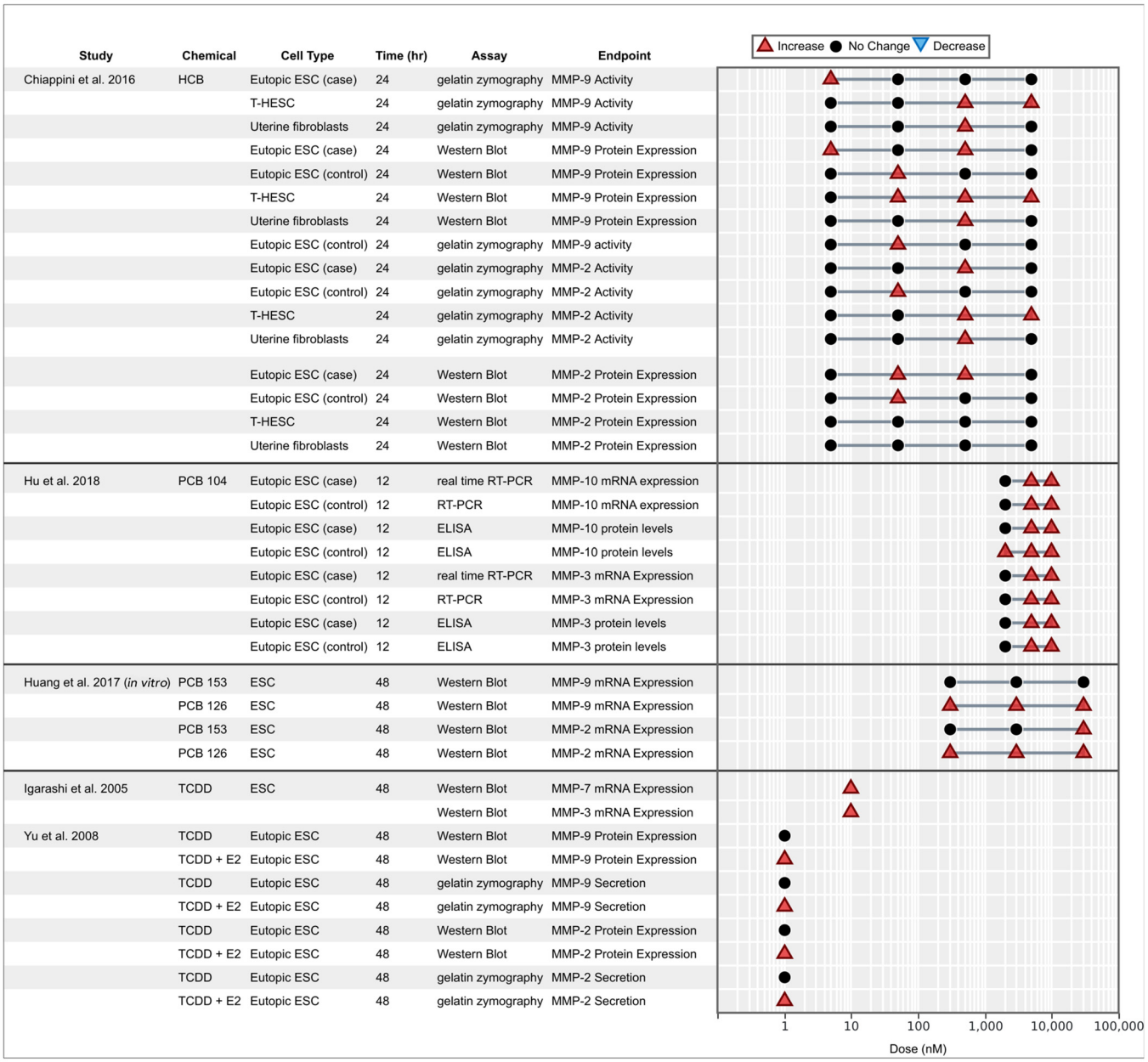


Figure 4. Associations between exposure to organochlorine chemicals and (A) migration/invasion and (B) molecular markers supporting migration/invasion reported from *in vitro* studies. Endometrial stromal cells (ESCs) marked with case or control indicate that the cells were derived from women with endometriosis (case) or without (control).

induce endometrial cannabinoid receptor type 1 (CB1-R) mRNA expression in control ESCs (treated with 1 nM E2; 500 nM P) (Resuehr et al. 2012). Steroid-induced expression of this gene was inhibited by cotreatment with 10 nM TCDD with or without IL-1α. TCDD exposure significantly blocked P induction of CB1-R mRNA (cannabinoid type 1 receptor) expression in control ESCs; the addition of IL-1α to TCDD had a synergistic effect in blocking this induction ($p < 0.001$) (Resuehr et al. 2012).

OCCs and aryl hydrocarbon receptor/aromatase/steroidogenesis axis. The AhR is a transcription factor that regulates gene expression and is involved in metabolism of xenobiotics. As an orphan nuclear receptor, its endogenous ligands are not known, and though some candidates have been suggested, the AhR remains most known perhaps for its role in binding with TCDD and other dioxin-like compounds. On activation, the AhR translocates into the cell nucleus and forms a dimer with the AhR nuclear translocator (ARNT), which leads to changes in gene transcription (Denison and Nagy 2003). We identified seven studies that measured AhR and AhR-related activities that may be relevant to endometriosis (Figure 7B). In endometrial explants, TCDD was found to increase both AhR and ARNT mRNA expressions (Pitt et al. 2001), but the relationship between TCDD and ARNT expression was mostly linked to covariates such as donor age and phase of the uterine cycle (Pitt et al. 2001). Yang (1999) reported similar results in ESC-SV40Ts: TCDD contributed to a significant increase in AhR mRNA expression with no impact on ARNT expression (Yang 1999).

Aromatase (CYP19A1), also known as estrogen synthase, is an enzyme responsible for a key final step in the biosynthesis of estrogens and is often regulated by the AhR (Lephart 1996). Aromatase is not normally expressed in healthy ESCs; elevated expression is thus associated with endometriotic diseases (Noble et al. 1996). This association is interesting in regard to the concomitant effect of TCDD and E2 on the pathogenesis of endometriosis (see above). Aromatase activity was found to increase significantly in granulosa cells at 1 and 10 μM ($p < 0.05$), but remained unchanged in ESCs after atrazine exposure (Holloway et al. 2008). DDE increased aromatase activity in ESCs ($p < 0.05$) at both 50 and 100 ng/mL (Holloway et al. 2005). Huang et al. (2017) did not observe a change in CYP19A1 mRNA levels in ESCs exposed to either PCB 126 or PCB 153, but found up-regulation of 17β-hydroxysteroid dehydrogenase 7



Study	Chemical	Cell Type	Time (hr)	Assay	Endpoint
Chiappini et al. 2016	HCB	Eutopic ESC (case)	24	gelatin zymography	MMP-9 Activity
		T-HESC	24	gelatin zymography	MMP-9 Activity
		Uterine fibroblasts	24	gelatin zymography	MMP-9 Activity
		Eutopic ESC (case)	24	Western Blot	MMP-9 Protein Expression
		Eutopic ESC (control)	24	Western Blot	MMP-9 Protein Expression
		T-HESC	24	Western Blot	MMP-9 Protein Expression
		Uterine fibroblasts	24	Western Blot	MMP-9 Protein Expression
		Eutopic ESC (control)	24	gelatin zymography	MMP-9 activity
		Eutopic ESC (case)	24	gelatin zymography	MMP-2 Activity
		Eutopic ESC (control)	24	gelatin zymography	MMP-2 Activity
		T-HESC	24	gelatin zymography	MMP-2 Activity
		Uterine fibroblasts	24	gelatin zymography	MMP-2 Activity
		Eutopic ESC (case)	24	Western Blot	MMP-2 Protein Expression
		Eutopic ESC (control)	24	Western Blot	MMP-2 Protein Expression
		T-HESC	24	Western Blot	MMP-2 Protein Expression
		Uterine fibroblasts	24	Western Blot	MMP-2 Protein Expression
Hu et al. 2018	PCB 104	Eutopic ESC (case)	12	real time RT-PCR	MMP-10 mRNA expression
		Eutopic ESC (control)	12	RT-PCR	MMP-10 mRNA expression
		Eutopic ESC (case)	12	ELISA	MMP-10 protein levels
		Eutopic ESC (control)	12	ELISA	MMP-10 protein levels
		Eutopic ESC (case)	12	real time RT-PCR	MMP-3 mRNA Expression
		Eutopic ESC (control)	12	RT-PCR	MMP-3 mRNA Expression
		Eutopic ESC (case)	12	ELISA	MMP-3 protein levels
		Eutopic ESC (control)	12	ELISA	MMP-3 protein levels
Huang et al. 2017 (*in vitro*)	PCB 153	ESC	48	Western Blot	MMP-9 mRNA Expression
	PCB 126	ESC	48	Western Blot	MMP-9 mRNA Expression
	PCB 153	ESC	48	Western Blot	MMP-2 mRNA Expression
	PCB 126	ESC	48	Western Blot	MMP-2 mRNA Expression
Igarashi et al. 2005	TCDD	ESC	48	Western Blot	MMP-7 mRNA Expression
				Western Blot	MMP-3 mRNA Expression
Yu et al. 2008	TCDD	Eutopic ESC	48	Western Blot	MMP-9 Protein Expression
	TCDD + E2	Eutopic ESC	48	Western Blot	MMP-9 Protein Expression
	TCDD	Eutopic ESC	48	gelatin zymography	MMP-9 Secretion
	TCDD + E2	Eutopic ESC	48	gelatin zymography	MMP-9 Secretion
	TCDD	Eutopic ESC	48	Western Blot	MMP-2 Protein Expression
	TCDD + E2	Eutopic ESC	48	Western Blot	MMP-2 Protein Expression
	TCDD	Eutopic ESC	48	gelatin zymography	MMP-2 Secretion
	TCDD + E2	Eutopic ESC	48	gelatin zymography	MMP-2 Secretion

Figure 5. Associations between exposure to organochlorine chemicals and expression and secretion of matrix metalloproteinases reported from *in vitro* studies. Endometrial stromal cells (ESCs) marked with case or control indicate that the cells were derived from women with endometriosis (case) or without (control).

(HSD17B7) after exposing the cells to the dioxin-like PCB126. These effects were apparently mediated by the AhR because the specific inhibitor 3′,4′-dimethoxyflavone (DMF) counterbalanced the effects. CYP1A1 and CYP1B1 are involved in endocrine modulation through the metabolic activation of polycyclic aromatic hydrocarbons and by metabolizing E2 to the 2- and 4-hydroxylated derivatives, respectively (Coumoul et al. 2001; Hayes et al. 1996; Safe 1995). TCDD exposure was found to significantly increase CYP1A1 expression in endometrial explants (Bofinger et al. 2001) and hTERT-EECs (Willing et al. 2011). EROD, a biomarker of CYP1A1 induction (Whyte et al. 2000), was found to increase significantly ($p < 0.00001$) in explants after exposure to TCDD (10 μM) and subsequent exposure for 48 h to hormones (10 nM E2, 500 nM P) (Bofinger et al. 2001). CYP1B1 was also found to increase in response to TCDD (Bofinger et al. 2001; Willing et al. 2011).

RoB Assessment of Individual Studies

The internal validity was assessed using the RoB tool developed by NTP/OHAT (NTP/OHAT 2015b) for each study that reported a primary outcome (16 *in vivo* studies and 9 *in vitro* studies) (Figure 8; percentage breakdown in Figure S7). Further details can be found online in HAWC for *in vivo* studies (https://hawcproject.org/study/assessment/812/rob-invivo/) and *in vitro* studies (https://hawcproject.org/study/assessment/812/rob-vitro/).

Most studies (80%, $n = 20$) reported randomization in exposure allocation, whereas the remainder did not report the methods used for study group allocation. Although no studies reported methods of randomization or blinding of allocation, author correspondence confirmed allocation concealment for 15 studies (60%). A total of 24 studies (84%) explicitly reported identical experimental conditions and treatment vehicles across study groups, and the same

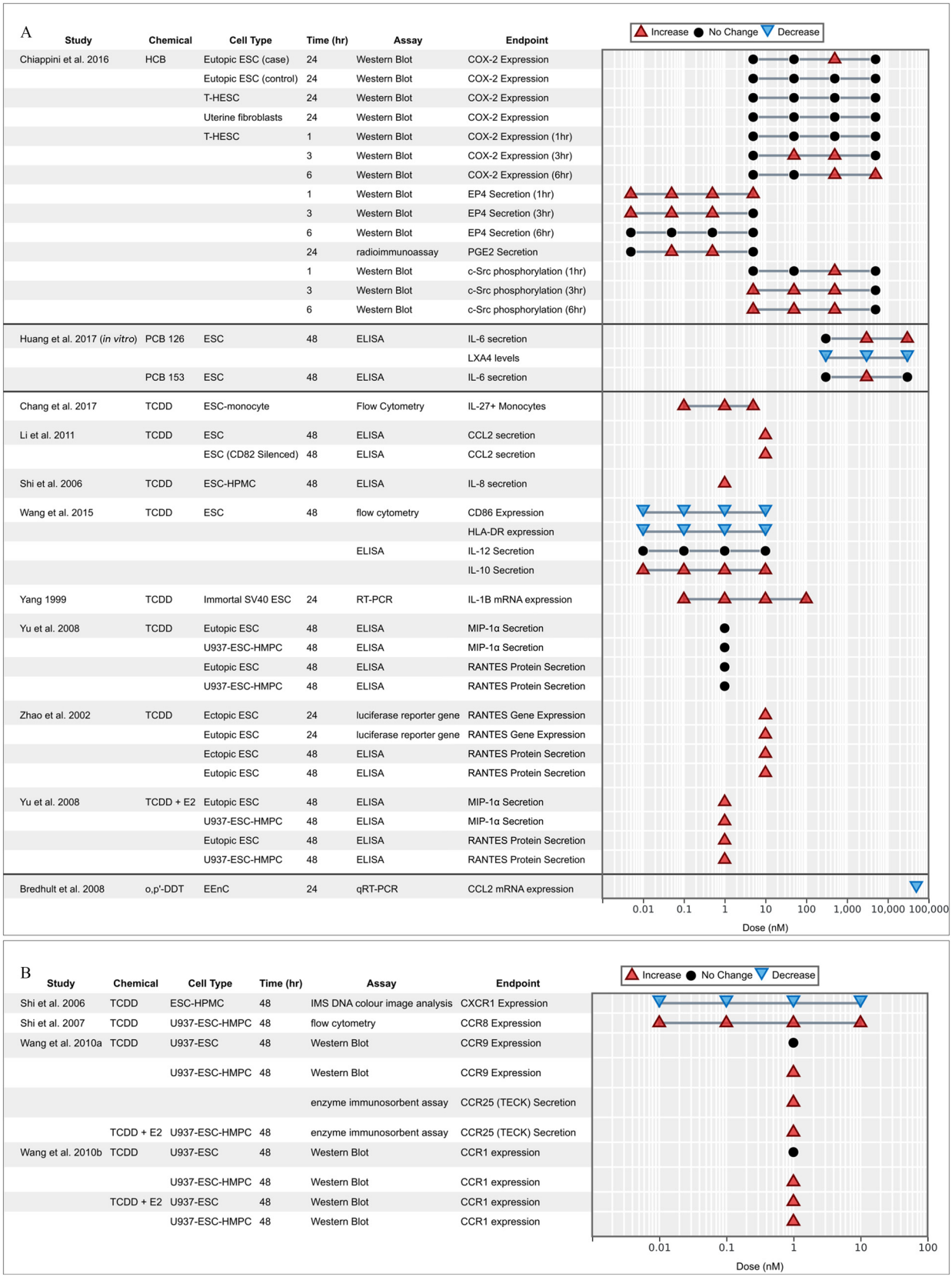


Figure 6. Associations between exposure to organochlorine chemicals and (A) chemokine expression and (B) their receptors reported from *in vitro* studies. Endometrial stromal cells (ESCs) marked with case or control indicate that the cells were derived from women with endometriosis (case) or without (control).

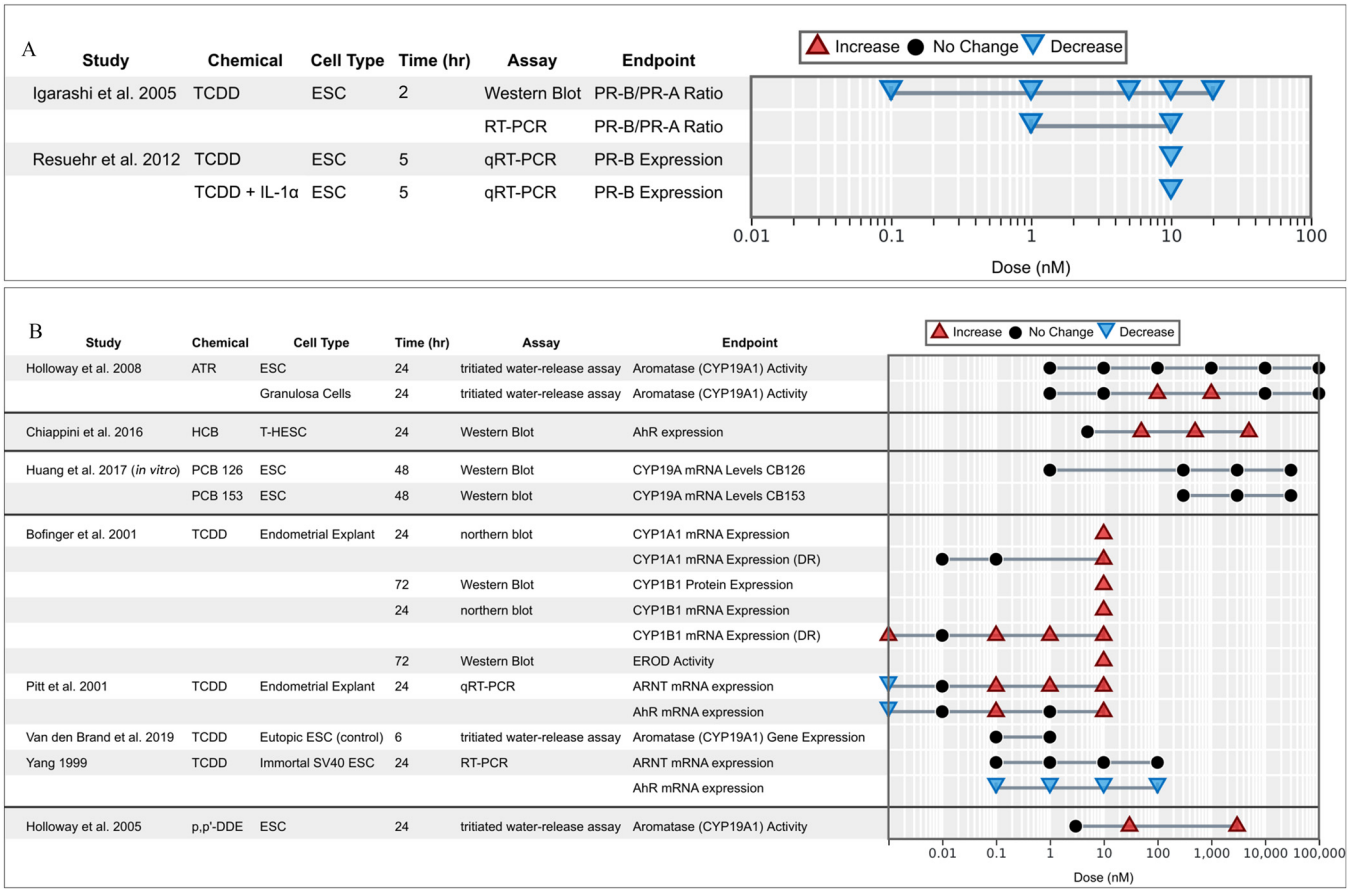


Figure 7. Associations between exposure to organochlorine chemicals and (A) the progesterone receptor and (B) AhR/ARNT and aromatase (CYO19A1) related activities and expression reported from *in vitro* studies. Endometrial stromal cells (ESCs) marked with case or control indicate that the cells were derived from women with endometriosis (case) or without (control).

proportion reported probably low or definitely low risk of attrition bias. Two studies explicitly documented the animals that died during the experiment and accounted for the deaths (Rier et al. 1993; Arnold et al. 1996). Research personnel were confirmed to be blinded to the study groups in fewer than half of the studies (44%, $n = 11$). Twelve studies (48%) provided the source and purity of the chemical exposures, although only the studies on rhesus monkeys performed an independent test for impurities and contaminants studies (Arnold et al. 1996; Rier et al. 1993). Nearly all (94%, $n = 15$) *in vivo* studies reported reliable outcome assessment. The gold standard for assessment of the presence of endometriosis or the growth of lesions is through laparoscopy and necropsy with measurement of lesions with calipers (Story and Kennedy 2004). Further confirmation performed through a histological examination made the difference between probably low and definitely low RoB. Almost all studies reported the measured outcomes expressed in the "Introduction" and "Methods" sections, with the exception of one review article that did not have a "Methods" section (Bruner-Tran et al. 1999) and another study that partially reported results (Nayyar et al. 2007). For other potential threats to internal validity, we considered whether or not the statistical analyses performed were appropriate, including a test for homogeneity of variances, the assumption necessary to reliably perform parametric tests.

Confidence Rating for the Body of Evidence

Additionally, a confidence rating was determined for each primary outcome and its association with TCDD exposure (Figures S8–S11) because TCDD was the most reported exposure in both *in vivo* and *in vitro* studies. Three key RoB criteria were used to categorize each primary outcome into three confidence tiers, which served as the basis of the level of evidence assessment (below). Among the *in vivo* studies reporting associations between TCDD and onset, one was classified in Tier 1 (Rier et al. 1993) (responses mostly "definitely low" and "probably low" for RoB) and two studies in Tier 3 (Bruner-Tran et al. 1999; Nayyar et al. 2007) (responses for RoB mostly "not reported" or "probably high" with some "definitely high") (Figure S8). Because 2 out of 3 of the studies ranked in Tier 3, suggesting "serious risk" of bias for this end point, confidence rating was downgraded for onset. The body of evidence on associations between TCDD and *in vivo* lesion growth were rated Tier 1 and Tier 2. The RoB for this end point was thus deemed "not serious," and the confidence rating was not downgraded (Figure S9). For *in vitro* migration/invasion, TCDD studies were rated either Tier 2 or Tier 3, mainly penalized by the underreporting of chemical standard details, blinding, or incomplete data, suggesting "serious risk" of bias (Figure S10). Confidence rating was thus downgraded for migration/invasion. Last, for *in vitro* viability/proliferation, TCDD studies were rated Tier 1 and Tier 2 (Figure S11). The RoB was deemed "not serious," and thus confidence rating was not downgraded for viability/proliferation.

In addition to the RoB analysis, we considered several other categories to downgrade or upgrade confidence for each of the four primary outcomes following TCDD exposure. The level of evidence assessment is summarized in Table 5. To assess indirectness, we considered first relevance of the models to human

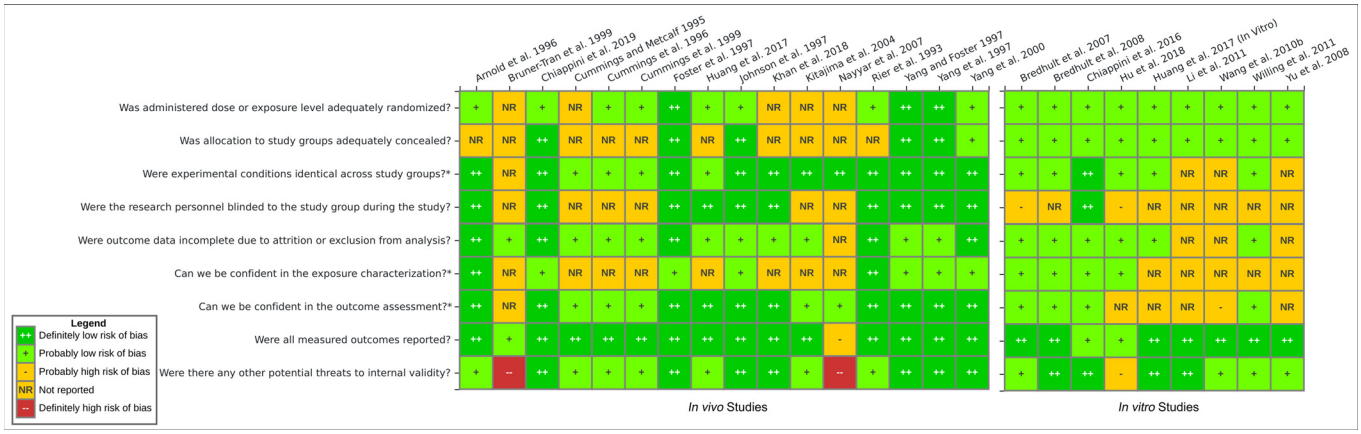


Figure 8. Risk of bias heatmap for the *in vivo* and *in vitro* studies that reported primary end points. Ratings were determined by two independent evaluators following the criteria adapted from OHAT RoB tool (NTP/OHAT 2015b). Additional details and justifications for each risk of bias rating are available online in HAWC interactive figures for *in vivo* studies (https://hawcproject.org/study/assessment/812/rob-invivo/) and *in vitro* studies (https://hawcproject.org/study/assessment/812/rob-vitro/). Note: HAWC, Health Assessment Workspace Collaborative; OHAT RoB, Office of Health Assessment and Translation Risk of Bias.

health. Among animal studies, nonhuman primate models are considered the most relevant due to their ability to menstruate and spontaneously develop endometriosis. Rodent models were thus less direct, because endometriosis does not spontaneously occur in rodents. Nevertheless, the development and contribution of rodent models toward the understanding of endometrial phenotypes, the peritoneal microenvironment, and the immune system has been extensively documented (Bruner-Tran et al. 2002, 2018; Vernon and Wilson 1985). For *in vitro* models, only noncancer human endometrial cell models or explants were included in this review, and thus all were considered relevant for humans. Additionally, tested doses in animal and *in vitro* studies were plotted to compare their relevance to human exposure levels. Dose levels of *in vitro* studies fell within the levels detected in humans in previous epidemiological studies (Figure S12). On the other hand, most *in vivo* animal studies used doses that were a few orders of magnitude higher than the highest human internal exposures. Previously published literature has found that animals and humans respond at similar body burdens, especially for dioxin-related effects like induction of CYP19A (Devito et al. 1995). However, differences between internal and external dosing and species variability in metabolism and toxicokinetics fall within the uncertainty range commonly acknowledged in risk characterization. Following the guidance of the *NTP/OHAT* handbook, there is no downgrading for dose levels used in experimental animal studies for confidence rating determination (NTP/OHAT 2015a). Considering these elements, no end points were downgraded for indirectness.

The consistency of results on *in vivo* onset and *in vitro* migration/invasion across study designs, models and doses merited an upgrade in confidence in those bodies of evidence where highly heterogeneity was expected. There were also inconsistencies for *in vivo* lesion size, but we found that these could be explained by differences in experimental design and animal models. Despite the often significant increases in lesion size, especially for murine models, in a monotonic dose–response manner (Cummings and Metcalf 1995b; Cummings et al. 1996; Johnson et al. 1997; Khan et al. 2018), the results were not consistent nor significant enough across all studies and models to merit an increase in confidence for dose response or magnitude of effect. Conversely, the divergence of results on viability/proliferation *in vitro* using similar doses and assays merited a downgrade in confidence due to unexplained inconsistency.

Considering the direction of health effects, we translated the confidence ratings into a level of evidence for health effect NTP/OHAT framework (Table S6) and displayed it in the Evidence Profile Table 5. For *in vivo* end points, there was high confidence in the body of evidence for health effect, translating to a high level of evidence linking TCDD exposure and increased endometriosis onset and lesion growth. For *in vitro* end points, there was high confidence for health effect on cell migration, translating to a high evidence linking TCDD exposure and increased endometrial cell migration/invasion. However, for endometrial cell viability or proliferation, there was moderate confidence in the body of evidence for no health effect, which translated to an inadequate level of evidence of no effect of TCDD on cell viability/proliferation (Table 5; Table S6). The evidence gathered from the rest of the OCCs further suggest a potential joint effect on cell migration, mainly by boosting the AhR/CYP1A1 pathway, promoting inflammation of the microenvironment or extracellular matrix-remodeling (Figure 9).

Epidemiological Evidence Integration and Hazard Identification

We previously established a moderate level of evidence for the associations between TCDD and dioxin-toxic equivalents and endometriosis in human epidemiological studies ($n = 10$), using the same evidence-based framework (Cano-Sancho et al. 2019). This was integrated with the high level of evidence established in the present systematic review for TCDD on related endometriosis outcomes (onset and lesion growth) in animal studies for a preliminary classification of "Presumed" hazard according the NTP/OHAT scheme (Figure S6). We then considered the different *in vitro*/mechanistic studies supporting the biological plausibility of such associations and consider some potential upgrading or downgrading. In this regard, although there was insufficient evidence of TCDD's effects on cell viability/proliferation in *in vitro* studies, the strong support from the combined high level of evidence found for TCDD on cell migration/invasion in *in vitro* studies and the consistent effects from the supportive secondary outcomes justified the upgrading of the hazard identification of TCDD as a "Known" hazard for endometriosis in humans.

It is important to consider that this component-based approach may not reflect the realistic effect of TCDD on endometriosis, considering that TCDD is commonly found in combination with a number of other co-occurring OCCs in humans. Hence, taking a more realistic mixture-based approach, this hazard identification conclusion is even further supported by the

Table 5. Evidence profile table for the associations between TCDD and the primary outcomes related to endometriosis.

Body of evidence	Initial rate of confidence	Downgrading factors					Upgrading factors			Final rate of confidence	Health effect[d]	Level of evidence
		Risk of bias[a]	Unexplained inconsistency[b]	Indirectness	Imprecision	Publication bias	Large magnitude	Dose–response	Consistency[c]			
Onset (*in vivo*)	High	Downgrade							Upgrade	High	Effect	High
Lesion growth (*in vivo*)	High									High	Effect	High
Migration/invasion (*in vitro*)	High	Downgrade							Upgrade	High	Effect	High
Viability/proliferation (*in vitro*)	High		Downgrade							Moderate	No effect	Inadequate

[a]Rating downgraded for risk of bias for onset and migration/invasion as the most studies ranked Tier 2 and Tier 3 due to underreporting on key elements (Figures S8–S11).
[b]Rating downgraded for unexplained inconsistency for viability/proliferation because the two studies that reported on TCDD and proliferation found opposing directions of effect, which could not be explained by study design (Figure 3).
[c]Rating upgraded for consistency for onset and migration/invasion because of consistent direction of effect across multiple study designs and animal/cell models (Figure 4).
[d]Evidence of health effect in onset, lesion growth, and migration/invasion. Evidence of no health effect in viability/proliferation. Based on this, determinations for the level of evidence were made (Table S6).

potential additive and synergistic effects of those co-occurring OCCs (e.g., PCBs, HCB), with analog dioxin-like and estrogenic activities. Nevertheless, the goal of this classification is not intended for regulatory purposes but rather to provide a robust evaluation of the state of the science that currently exists for TCDD and dioxin-like chemicals on endometriosis outcomes. For the rest of the OCCs, we cannot establish conclusions in a component-based manner because the level of animal and *in vitro* evidence was considered inadequate.

Discussion

To the best of our knowledge, this is the first study to systematically gather and synthesize the experimental evidence linking OCC exposure to endometriosis and endometriosis-related outcomes. Most evidence is associated with TCDD exposure, which appears to consistently contribute to outcomes directly related to endometriosis, including *in vivo* lesion growth or *in vitro* invasiveness. Inflammation and extracellular matrix remodeling appear to be critical intermediary end points, which are disrupted by other OCCs, such as PCB126 or HCB, as well. The level of evidence was determined to be high for the associations of TCDD exposure on *in vivo* onset and lesion growth and *in vitro* cell migration/invasion. The level of evidence was deemed inadequate to conclude that TCDD has no effect on *in vitro* proliferation.

In this review, we also curated an assembling of evidence to facilitate a more robust dissertation on the potential mechanisms of action underlying the development of the disease linked to the exposure to OCCs. Inspired by the adverse outcome pathway (AOP) concept, we have organized the evidence in primary (i.e., adverse outcomes) and secondary end points (i.e., key events) that are initiated by various exposure and endogenous hormones (i.e., stressors), allowing the networking representation of intermediary and interrelated associations (Carvaillo et al. 2019). This framework also aligns with the "Key characteristics" approach proposed for carcinogens, endocrine disruptors, or reproductive toxicants (Guyton et al. 2018; La Merrill et al. 2020; Luderer et al. 2019). The ability of chemicals to alter some or all of the primary and secondary end points may help shed some light on the simultaneous effect of chemicals in realistic mixtures as commonly reported in epidemiological studies. In the synthetic proposed framework, we assume the oversimplification of the complex physiology of endometriosis, which has at least three phenotypically distinct subtypes (deep infiltrating, ovarian, and peritoneal/superficial) in humans, and only one of which (peritoneal) has been studied in these experimental models. Nonetheless, moving forward, we can see the potential of expanding the linear frameworks traditionally applied in hazard identification toward a network architecture. At the same time, the systematically gathered evidence in the present work may urge systems toxicologists to complete the much-needed AOP, initiated by stressors like environmental chemical exposures that potentially lead to endometriosis.

Despite the heterogeneity of the examined literature corpus, the reviewed data considered together suggest that TCDD and other dioxin-like chemicals are of particular concern in the pathogenesis and development of endometriosis. The role of AhR, commonly found at higher concentrations in endometriotic tissue than in healthy eutopic tissue, appears of major relevance to mediate the effects of dioxins and dioxin-like compounds such as PCB126 or HCB. In the case of HCB, two pathways were proposed involving *a*) the binding of HCB with the AhR-c-SRc complex triggering the c-Src phosphorylation and subsequent stimulation of growth factors receptors and activation of MMPs, and *b*) the translocation of HCB-AhR complex to the nucleus leading COX-2, VEGF and TNF-α expression, subsequent PGE_2

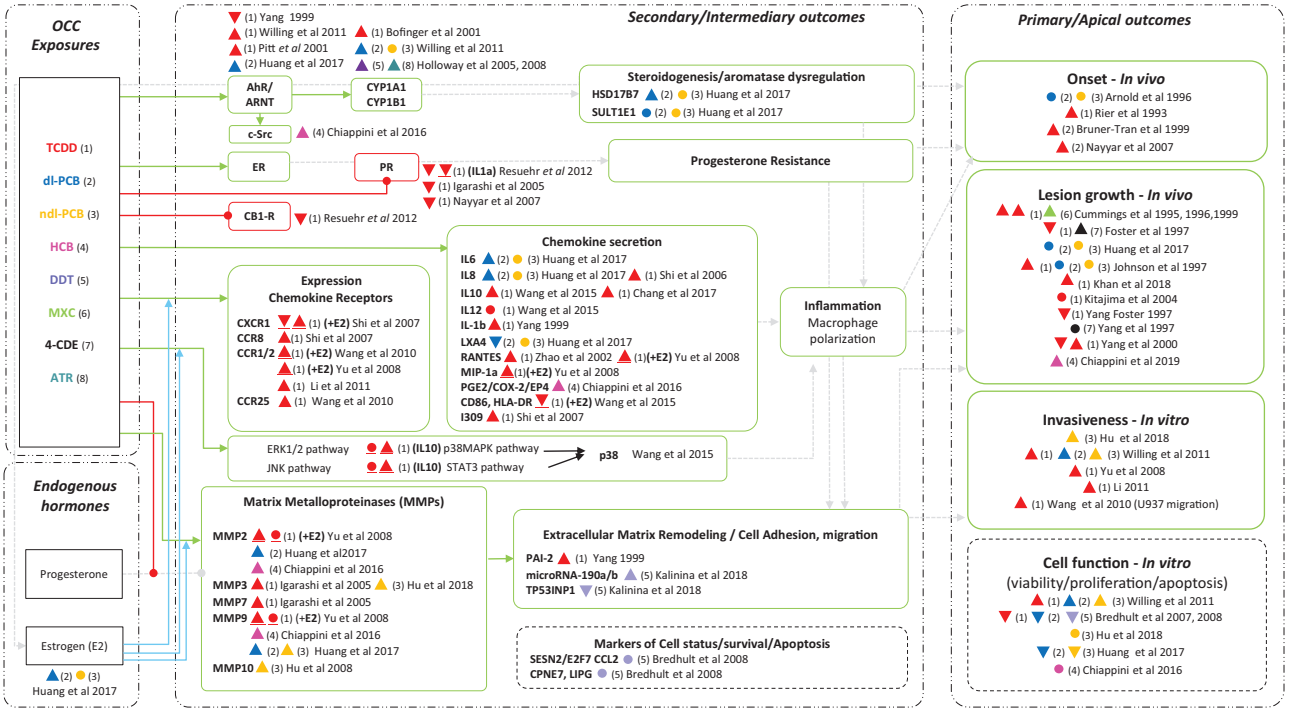


Figure 9. Network evidence plot summarizing the associations between exposure to organochlorine chemicals (OCCs) and the different end points related to endometriosis. Triangles and circles summarize the overall effect direction of the tested end point relative to the control. The triangle indicates significant increase, upside-down triangle significant decrease and the circle no significant effect. The colored number key in parenthesis identifies the specific OCC indicated in the "Exposure box," also identified by the number in parenthesis [e.g., "red triangle (1)" indicates significant increase for TCDD]. In case of coexposure (i.e., with E2), the symbol appears underlined. Connecting lines ending with arrows indicate the overall evidence suggests up-regulation/increase, whereas connecting lines ending with circles indicate down-regulation/inhibition. Solid blue arrows indicate the presence of interactions, and dotted gray arrows stand for inferred associations between end points on the basis of current knowledge. The contour of end point boxes relates to the overall direction of effect toward positive associations (e.g., continuous line) or no conclusive effect (broken line). Note: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; ATR, atrazine; CB1-R, cannabinoid receptor type 1; CCR, chemokine receptor; COX, cyclooxygenase; CYP1A1, cytochrome P450; ER, estrogen receptor; HCB, hexachlorobenzene; IL, interleukin; LX, lipoxin; MXC, methoxychlor; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PCB, polychlorinated biphenyl; PGE, prostaglandin E; PR, progesterone receptor.

synthesis, and stimulation of EP4 signaling, culminating with the activation of c-Src and MMPs (Chiappini et al. 2016, 2019). Dioxin-like PCBs were found to increase HSD17B7 expression through both decreasing HSD17B7 promoter methylation and by decreasing LXA4, which subsequently also increases inflammation (Huang et al. 2017). HSD17B7 is also involved in estrogen biosynthesis, and there is some evidence of increased expression of 17β-HSDs in endometriotic lesions (Delvoux et al. 2014) as well as in endometrial tumors and carcinomas (Lax 2017). The concomitant effect of OCCs on the activation of aromatase and steroidogenesis with the effects on P resistance may suggest the presence of a positive feedback loop promoting the activation of MMPs and proinflammatory microenvironment, facilitating the adhesion and migration of cells, translating to lesion growth in the animal models. P and retinoic acid, which is degraded by CYP1 induced by TCDD-activated AhR, are found to be natural inhibitors of endometrial MMP expression (Bruner-Tran et al. 1999) and may mitigate development of endometrial lesions. TCDD was found to inhibit P's suppression of MMP expression, thus promoting endometrial lesion proliferation (Bruner-Tran et al. 1999). TCDD was also found to block P-mediated induction of TGF-β2, a necessary paracrine signal to inhibit MMP expression (Bruner-Tran et al. 1999). P- and TCDD-treated mice were thus unable to inhibit MMP suppression and the development of endometriotic lesions. Expression of P-induced TGF-β2 was also found to be significantly decreased in the uteri of mice maximally exposed to TCDD (Nayyar et al. 2007). *In vitro* studies in this review have identified important cross talk between macrophages and ESC co-cultures exposed to TCDD and E2, providing evidence for the role of M2 macrophage activation by chemokines in promoting endometriosis; exposure to TCDD, especially in combination with E2—the synthesis of which is linked to aromatase—contributes to increased invasion in ESCs co-cultured with HMPCs, U937 cells, and/or macrophages, more than in ESCs cultured alone (Chang et al. 2017; Li et al. 2011; Shi et al. 2007; Wang et al. 2010b, 2015). One implication of macrophages not explored by the studies in this review are the neuroimmune interactions between macrophage-induced stimulation of inflammatory cytokines and peripheral nerves (Liang et al. 2018). These neuroinflammatory macrophage–nerve interactions have been strongly tied to endometriosis-related pain (Forster et al. 2019; Wu et al. 2017).

Sources of Heterogeneity

Experimental models. As previously mentioned, a number of factors posed a challenge to evidence synthesis as sources of heterogeneity between studies. First, there were considerable differences in the various models used to study endometriosis. Within animals, three species of animal models were identified: monkey, mouse, and rat. Differences between rat and mouse models varied sometimes from magnitude of effect to even direction of effect (Cummings et al. 1996), suggesting there are species differences in the mechanisms mediating TCDD's actions in promoting

endometriosis. Indeed, even within a single species, there was potential for model variation. The majority of mouse studies included in this review were performed with induction of endometriotic lesions through the autologous implantation of the uterine horn method developed by Vernon and Wilson (1985) in rats and later adapted to mice by Cummings and Metcalf (1995a). However, one study used a nude mouse model (Bruner-Tran et al. 1999), a method also described by Bruner-Tran et al. (2002). Unlike the induced lesion model, the immunocompromised nude mouse model allows the establishment of endometriosis-like growth of human endometrium and endometriotic tissues in mice introduced to human tissues and OCC exposures. Because the endometriotic lesions or phenotypes develop "spontaneously" in these models, "onset" of endometriosis can be measured. No studies have been published systematically comparing these two models. There is also no such comparison between primate and rodent models, but it can be assumed that there may be differences in how these animals respond to OCCs. One study compared a human *in vitro* endometrial model with a rat *in vitro* endometrial model (van den Brand et al. 2019). Results showed clear species differences in AhR, PR, and estrogen receptor (ER)-α in response to hormones, warranting more research into the applicability of certain animal models for endometriosis research. Nonetheless, models in which the spontaneous development of endometriosis or endometriosis-like phenotypes can occur are still a valuable source of information (Arnold et al. 1996; Bruner-Tran et al. 2002; Nayyar et al. 2007; Rier et al. 1993).

In *in vitro* studies, there was an even greater variety of cell populations used. The majority of studies used ESCs, including ESCs from both ectopic and eutopic endometriotic tissues, as well as eutopic ESCs from women with and without endometriosis. Differences in gene expression linked to infiltration and metastasis were found in eutopic vs. ectopic ESCs (Li et al. 2011) and in MMP expressions in the eutopic ESCs of control women vs. from women with endometriosis (Hu 2018). These examples highlight subtle but important differences in the variations of even the same cell type models. As expected, we also observed differences between cell models. Aromatase (CYP1A1) is not typically expressed in normal healthy ESCs and was thus not found to be affected in ESCs but was elevated in ectopic ESCs and granulosa cells (Holloway et al. 2005, 2008; Huang et al. 2017). Chemokine CCL2 increased in ESCs (Li et al. 2011) but decreased in EECs (Bredhult et al. 2008). It is hypothesized that this is because EECs express ER-beta (Critchley et al. 2001; Krikun et al. 2005) and are thus likely not to detect effects mediated by ER alpha. The roles each cell type play thus are likely to influence the results observed. Three studies used unique immortalized cell lines (Chiappini et al. 2016; Willing et al. 2011; Yang 1999), which sometimes displayed different characteristics than their nonimmortalized counterparts. For example, proliferative activity was found to significantly decrease in primary cultures of EECs (Bredhult et al. 2007, 2008) but had the opposite effect in hTERT-EECs (Willing et al. 2011); it is hypothesized that this effect difference is because TCDD has no antiestrogenic effects in hTERT-EECs. Last, several studies used cell co-cultures in different combinations (often combinations of ESCs, macrophage U937 cells involved in monocyte differentiation, and HMPCs that synthesize IL-6). Invasiveness increased in the three cell U937-ESC-HMPC co-cultures treated with TCDD but not in ESCs tested alone (and in neither of the other cell types tested alone) (Wang et al. 2010b). Several cytokine receptors (CCR1, CCR8, and CCR9) experienced similar effects (Shi et al. 2007; Wang et al. 2010b). This finding is evidence for cross talk between different cell types and highlights the importance of taking into consideration the complexity of these underlying cellular interactions in the real human system.

Exposures. The articles included in this review measured 14 unique OCCs, but most studies ($n = 27$) measured exposure to TCDD. Some used TCDD alone, and others tested in combination with hormones like E2 or P. Often, the addition of E2 created a positive synergistic effect (Li et al. 2011; Wang et al. 2010b; Yu et al. 2008). Other studies compared dioxin-like chemicals with nondioxin-like chemicals (Huang et al. 2017; Johnson et al. 1997). Although results varied in magnitude, they generally supported increases in onset and lesion growth in response to TCDD but less consistent for the other families. One of the most prominent nonhuman primate studies was conducted with the technical mixture Aroclor 1254 (Arnold et al. 1996), the exact composition and related toxicological effects of which have been shown to vary between lots and thus complicate the generalization of effects for individual congeners (Kodavanti et al. 2001). The specific Aroclor 1254 composition of that study was detailed in a previous report showing major concentrations for PCB77 (500 μg/g), PCB126 (100 μg/g), and PCB169 (1 μg/g), and because PCDDs are not detected at 0.1 μg/g (Mes et al. 1989), that could help to explain the divergent results with Rier et al. (1993).

For animal studies, other elements associated with dosing regimens were also significant sources of heterogeneity, including life stage assessed, overall study duration, and route of exposure. Most studies included regimens that were chronic (multiple years for the monkey studies and several months for rodent studies); two rodent studies used developmental regimens, and other studies included subchronic or acute findings with a single dose. These multitudinous variations make it difficult to assess total final internal doses in the animals, unless serum concentrations of the exposures were measured. In the follow-up study after the original chronic TCDD treatment on rhesus monkeys (Rier et al. 1993), authors correlated serum levels of TCDD with serum levels of multiple unintended PCBs and other polyhalogenated aromatic hydrocarbons accumulated through diet which were also associated with the presence of endometriosis (Rier et al. 2001). The findings from this follow-up also suggest the presence of synergistic mechanisms of organochlorines through the alteration of normal dynamics of accumulation of OCCs, which may be extended to other non-OCCs with metabolic disruption potential.

In many studies the animals were pretreated with E1 or E2 to encourage lesion survival. TCDD has known antiestrogenic effects, so in two studies, TCDD treatment actually significantly decreased lesion size in animals that had been pretreated with a high dose of estrogenic compounds [i.e., E1 (20 μg/kg/d) or E2 (24 ng/kg/d), respectively] (Foster et al. 1997; Yang and Foster 1997). Another study, which tested E2 treatment alone and in combination with TCDD, as well as TCDD alone without E2, found results that corroborate TCDD's antiestrogenic effects (Kitajima et al. 2004). Interestingly, one group studied the effects of two different vehicle controls (Khan et al. 2018). Though TCDD treatment in both corn oil and DMSO both significantly increased lesion size in comparison with their vehicle controls, authors found that TCDD + DMSO increased lesion size more than TCDD + oil, suggesting that the vehicles selected may have an impact on the magnitude of effects observed.

Research Gaps

One limitation discussed above is the heterogeneity across studies included. In particular, there is a dearth of studies on other OCCs besides TCDD. Only 3 out of the 14 OCCs were investigated in more than two studies. Another three OCCs were investigated in two studies and the rest only in a single study. The current paradigm still focuses on singular exposure–outcome pairings, which fails to take into consideration the reality of the complex

exposure mixtures to which humans are actually exposed. The strong correlation profiles of OCCs in epidemiological studies highlights that exposures to individual organochlorines are extremely unlikely (Cano-Sancho et al. 2018). We thus encourage future studies to explore mixed exposures or assessments of families of chemicals based on structural or biological similarities. For instance, there is evidence supporting the role of the estrogenic plasticizer bisphenol A (BPA) on the spontaneous development or progression of endometriosis lesions in rodents (Jones et al. 2018; Signorile et al. 2010). Thus, it may be speculated that synergistic interactions between pollutants like TCDD and nonpersistent estrogen-mimicking chemicals like BPA may occur as observed with E2.

The RoB assessment revealed a general lack of reporting of some key study features, including methods of randomization and blinding of research personnel to study group allocation, treatment throughout the study, and outcome assessment. A challenge in all controlled *in vitro* and *in vivo* studies is determining final total exposure or internal doses. Only one study verified dose content in the feed using gas chromatography and mass spectrometry over the 4-y treatment period and later performed a follow-up study that measured internal exposure levels in serum (Rier et al. 2001). Physiologically based toxicokinetic (PBTK) modeling would be needed to fill this lack of necessary experimental *in vivo* and *in vitro* data to calculate internal doses or dose relevance to humans more accurately. Although we acknowledge these lengths are often not feasible, we encourage researchers to be as thorough as possible in their study design, verification, and reporting methods. The RoB assessment of *in vitro* studies is a nascent field, and no tool currently exists specifically tailored to this capacity. We employed the RoB Tool set forth by NTP/OHAT, which was adapted for human and animal studies (NTP/OHAT 2015b; Rochester and Bolden 2015; Rooney et al. 2014) and *in vitro* studies during the evaluation of immunotoxicity of perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) (NTP 2016). However, we encourage future reviews to continue to explore different methods of assessing internal validity for *in vitro* studies.

Conclusion

The literature corpus included in the present review support the contribution of TCDD and other dioxin-like chemicals to the progression of endometriosis. Specifically, there was a high level of evidence of TCDD's effects on endometriosis onset and lesion growth *in vivo* and on cell migration/invasion *in vitro*. Inflammation and extracellular matrix remodeling appeared to be the most likely mechanisms boosting adhesion, migration, and invasion of endometrial cells by OCCs. This conclusion, however, should be understood in the context of the limited number of available studies, considerable heterogeneity in the included studies, and the limitations in data reporting and evidence appraisal. These findings are consistent with an analogous systematic review and meta-analysis performed on the same family of chemicals in epidemiological studies (Cano-Sancho et al. 2019). Additionally, we provide an extended AOP-inspired framework to integrate multiple end points and intermediary endometriosis-related outcomes, which may support the understanding of the simultaneous effects of multiple chemical exposures. For instance, other endocrine-disrupting chemicals, such as bisphenol A, phthalates, or perfluoroalkyl acids may indirectly contribute to the global effects of OCCs on endometriosis progression, targeting some of the characterized intermediary outcomes (La Merrill et al. 2020). Overall, we deemed the level of evidence for the associations between OCC exposure and endometriosis outcomes to be high, with a particular focus on TCDD and dioxin-like chemicals. The integration with a moderate level of epidemiological evidence brought us to conclude that TCDD was a known hazard for endometriosis in humans, strongly supported by mechanistic *in vitro* evidence, according the NTP/OHAT framework. In any case, more experimental research is needed to fill the gaps in understanding the relationships between OCCs and endometriosis.

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Simultaneous exploration of nutrients and pollutants in human milk and their impact on preterm infant growth: An integrative cross-platform approach

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ABSTRACT

Early nutritional management including fortified human breastmilk is currently recommended to fulfil the energy demands and counterbalance risks associated to preterm birth. However, little is known about the potential adverse effects of exposure to persistent organic pollutants (POPs) carried in human milk on preterm infant growth. We conducted a pilot study proving the application of an integrative analytical approach based on mass spectrometry (MS) coupled to advanced statistical models, favouring the comprehensive molecular profiling to support the identification of multiple biomarkers. We applied this workflow in the frame of a preterm infants' cohort to explore environmental determinants of growth. The combination of high resolution gas and liquid chromatography MS platforms generated a large molecular profile, including 102 pollutants and nutrients (targeted analysis) and 784 metabolites (non-targeted analysis). Data analysis consisted in a preliminary examination of associations between the signatures of POPs and the normalised growth of preterm infants, using multivariate linear regression adjusting for known confounding variables. A second analysis aimed to identify multidimensional biomarkers using a multiblock algorithm allowing the integration of multiple datasets in the growth model of preterm infants. The preliminary results did not suggest an impairment of preterm growth associated to the milk concentrations of POPs. The multiblock approach however revealed complex interrelated molecular networks of POPs, lipids, metabolites and amino acids in breastmilk associated to preterm infant growth, supporting the high potential of biomarkers exploration of this proposed workflow. Whereas the present study intended to identify simultaneously pollutant and nutrient exposure profiles associated to early preterm infant growth, this workflow may be easily adapted and applied to other matrices (e.g. serum) and research settings, favouring the functional exploration of environmental determinants of complex and multifactorial diseases.

1. Introduction

Preterm infants are at higher long-term health risks, including postnatal growth failure. Postnatal nutrition thus becomes a major strategy to counterbalance the associated deleterious effects (Agostoni et al., 2010; Boquien, 2018). Early nutritional management during the first weeks of life, including parenteral and enteral feeding with human fortified milk, has been recommended, particularly for very preterm infants, by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (EPSGHAN) to improve the infant development and cognitive function (Agostoni et al., 2010). Human breastmilk is a highly complex and dynamic media, characterized by a rich composition in energy and macronutrients, but also vitamins, minerals, immunoglobulins and enzymes. Energy intake has been identified as a main independent factor associated with infant growth until the age of 2 years (Hiltunen et al., 2018), being lipids the main contributor of

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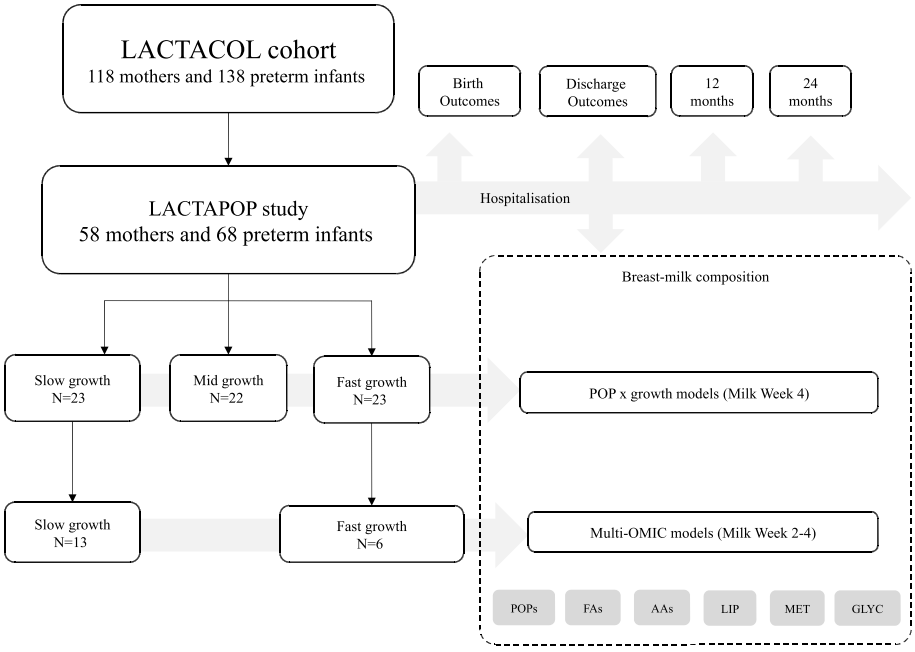


Fig. 1. Study design and sampling framework. Abbreviations: POPs, persistent organic pollutants; FAs, targeted fatty acids; AAs, targeted amino acids; LIP, non-targeted lipidome; MET, non-targeted metabolome; GLYC, non-targeted glycome.

energy requirements of healthy infants supplying up to 45–55%. The lipid fraction of breastmilk also represents a substantial source of functional and structural lipids, such as long-chain polyunsaturated fatty acids (LC-PUFA), derived from essential fatty acids: linoleic acid (LA) and α-linolenic acid (ALA), with central roles in brain development and child growth.

The nutritional richness of breastmilk is tightly confronted by the unintended presence of xenobiotics including the lipophilic pollutants excreted within the lipid fraction, such as the vast family of persistent organic pollutants (POPs). For these pollutants, toxicologically safe levels through breastfeeding have been proposed. For instance, a tolerable daily intake levels of 1–4 pg toxic equivalents (TEQ)/kg body weight (bw) per day have been proposed for polychlorinated dibenzo-dioxins and dibenzofurans (PCDD/F) (WHO, 2000; 2002), and a minimum risk level (MRL) is 30 ng/kg bw per day for polychlorinated biphenyls (PCBs) as group (ATSDR, 2000). The last report from the World Health Organization (WHO) United Nations Environment Programme (UNEP) global monitoring survey, revealed the still pervasive occurrence of POPs in breastmilk worldwide with highest levels of PCDD/Fs and PCBs in India, but also in some European countries (van den Berg et al., 2017). In all evaluated countries, infant exposure to dioxin-like TEQs was estimated to exceed by one or two orders of magnitude the safe levels, derived from perinatal effects reported in rodents and monkeys (van den Berg et al., 2017). The risk-benefit analysis associated to breast milk consumption emphasized that adverse health effects of POPs in milk appear mostly subtle and transient in nature, whereas the benefits of breastfeeding are largely supported by compelling evidence associated to reduced mortality and morbidity in preterm infants with a large list of health benefits during the entire life (van den Berg et al., 2017). In any case, no formal quantitative risk-benefit analysis of breastfeeding has been performed at individual level addressing the main methodological challenges and uncertainties associated to POPs in breastmilk (Boue et al., 2018; Nauta et al., 2018).

Considering the relevance of breastfeeding in the nutritional care of preterm infants and the tight relationship between lipid/energy fraction of breastmilk and POPs, we hypothesized that POPs may be particularly associated with the different breastmilk nutrient profiles and potentially impact preterm infant growth. Hence we conducted a proof-of-concept study illustrating the application of an integrated targeted and non-targeted analytical platform for the characterization of breastmilk pollutants, nutrients and metabolites, coupled to multidimensional statistical models to identify relevant molecular signatures for preterm infant growth and their associated networks. Specifically, the sub-objectives of the pilot study were: 1) to evaluate the associations between the concentrations of POPs and nutrient profiles (i.e. nutriome) in preterm breastmilk; 2) to evaluate the associations between the concentrations of POPs in breastmilk and early growth of preterm infants; and 3) to identify integrated signatures of POPs and nutrients in human milk associated to the early growth of preterm infants accounting for the underlying correlation structures.

2. Material and methods

2.1. Study population

The present pilot study was conducted on a sub-group of participants selected among the mother-preterm infants' dyads enrolled in the mono-centric prospective population-based LACTACOL birth cohort (registered at www.clinicaltrails.gov as #NCT01493063) recruited from October 2011 to March 2016 in the University Hospital of Nantes, France. The milk biobank was approved by the Committee for the Protection of Persons in medical research (CPP CB-2010-03). A written consent was obtained from all participants at enrolment. One hundred thirty-eight pre-term infants with no major congenital disease except prematurity, were included, for a total of 118 mothers finally enrolled in the LACTACOL cohort. Further details of the cohort have been published elsewhere (Alexandre-Gouabau et al., 2018). From the entire cohort, two overlapping sub-cohorts were created for this proof-of-concept study. For the first sub-cohort (LACTAPOP), a random subsample of 58 of mothers (delivering 68 infants, including 10 twins, born between 28 and 34 weeks of gestational age) where selected to conduct the present study involving the determination of POPs levels (Fig. 1). The selection of participants was conducted without any knowledge of exposure levels and with no formal sample size calculation due to the exploratory nature of this pilot study. The 68 selected infants were ranked according to their change in weight Z-score (expressed in units of Standard Deviation (SD) and calculated as previously described (Alexandre-Gouabau et al., 2018), depicting their growth velocity

during the hospital stay (i.e. difference between weight z-scores at birth and discharge) and then, were grouped in tertiles of their delta weight z-scores. For the second sub-cohort (i.e. cross-platform approach), a small group was previously selected with infants exhibiting extreme patterns of postnatal growth, belonging to the first and third tertile of weight Z-score from the entire LACTACOL cohort distribution (Alexandre-Gouabau et al., 2018). To sum up, from the preliminary selection of 26 infants (n = 11, "fast growth" and n = 15 "slow growth"), a subgroup of 19 infants (n = 6, "fast growth" and n = 13 "slow growth") were finally included in this pilot study because complete data on POPs and nutrients in milk was available (Fig. 1).

Infants admitted to the Neonatal Intensive Care Unit at Nantes University Hospital were eligible if they received human milk as their sole feeding for more than 28 days. Clinical characteristics were collected both on mothers and infants, including: maternal age, educational level, pre-gravid BMI, adverse events during pregnancy and delivery, infants' gestational age, birth weight and head circumference, growth trajectory through hospital discharge, and events during hospital stay in neonatology (Cohort characteristics in Supplemental Table 1). Volume of milk delivered per feeding session, fortifiers used and fortification level included in the parenteral nutritional supply (around 10 kcal/kg body weight/day), that generally does not exceed the first two weeks of life, as well as enteral intake, predominantly with expressed own mother's breast milk, was recorded daily. Energy and protein contents in preterm standard formula were 72 kcal/100 mL and 2 g/100 mL, respectively. Preterm infants received parenteral nutrition and minimal enteral feeding, predominantly with expressed breast milk, for a minimum of two weeks. By taking into account native own mother's milk macro-nutriments contents and routine fortification, the mean total enteral intake, particularly total energy intake, were reasonably close to that of EPSGHAN guidelines regarding daily and macronutrient intake, except for protein intake (which was 28% below recommended intake) (Alexandre-Gouabau et al., 2018). The median energy intake was 124 kcal/kg body weight/day in both, the "faster growth" and the "slower growth" group, being 104 kcal/kg body weight/day in the "medium growth" group (p-value = 0.29). Anthropometrical evaluation of infants was registered at follow-up visits conducted at 12 and 24 months.

2.2. Human milk collection

Breast milk expression was performed manually by mothers at home, using a Medela Manual Breast pump (Medela Inc., Etampes, France). Weekly representative 24-h mature milk samples were obtained by pooling breast milk sampled from 5 to 6 bottles brought daily to the Milk Bank of the Nantes University Hospital. The whole milk pool was homogenized with a disruptor (Polytron, Lucerne, Switzerland) and kept frozen at 80 °C until untargeted and targeted analysis of nutriome at week 2 and week 4 of lactation. Preliminary analysis using principal components showed no marked trajectories on nutrients during the hospital stay, supporting the use of these time points. A 30 ml-breast milk expression was specifically collected at once, at discharge and frozen at −80 °C until submitted to the analytical framework (See Fig. 2).

2.3. Breast-milk lipidomic profiling

2.3.1. *Breast milk Liquid Chromatography-High-Resolution-Mass Spectrometry (LC-HRMS)–based lipidomic (LIP), metabolomic (MET) and glycomic (GLYC) untargeted profiling*

Milk aliquots (50 μl) were submitted to the following preliminary treatment before analysis: 50 μl of sodium chloride 150 mmol/L were added to each milk sample followed by 100 μl of methanol and then, 100 μl of chloroform, according to a modified "Bligh-Dyer" extraction. Then, two extractions with 100 μl chloroform were performed. After centrifugation (10,000 g for 10 min), the organic and aqueous layers were collected separately, dried under N2and subsequently reconstituted, in acetonitrile-isopropanol-water (ACN: IPA: H_2O 65:30:5, v/v/v), and in water-acetonitrile (H2O: ACN 95:5, v/v), for lipid (lipidome) and polar (metabolome) species, respectively. A 1200 infinity series® high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, California, USA) coupled to an Exactive Orbitrap® mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for both lipidomic and metabolomic profiling as previously described (Alexandre-Gouabau et al., 2019). Briefly, lipid species separation was performed on a reverse phase CSH® C_{18} (100 × 2.1 mm^2 i. d., 1.7 μm particle size) column (Waters Corporation, Milford, MA). Sample metabolomics fingerprinting was performed, on the same LC-HRMS system, on a reverse phase with a Hypersil GOLD C18 column (1.9 μm particle size, 100 × 2.1 mm). The precision associated with sample preparation and LC-HRMS measurement was determined on the basis of a quality control (QC) consisting of a pool of 10 mothers' milk provided by the milk bank of Nantes Hospital Center. QCs samples were extracted similarly than others samples and injected sequentially in-between the milk samples. The tight clustering of QC samples before and after normalization were checked on principal component analysis (PCA) score plot. The extraction and reduction of human milk oligosaccharides (HMO) were performed as previously described, following milk delipidation by centrifugation and NaBH4-reduction (Alexandre-Gouabau et al., 2019; Oursel et al., 2017). Milk reduced oligosaccharides were separated on a Hypercarb® column (2.1 mm i. d. × 100 mm, 3 μm particle size, Thermo Scientific, San Jose, CA, USA) on an Ultimate 3000 HPLC system coupled to an LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA).

2.3.2. *Lipidomic, metabolomic and glycomic data analysis and metabolites characterization*

As previously described (Alexandre-Gouabau et al., 2018, 2019), lipidomics and metabolomics raw data files were preprocessed with Xcalibur 2.2® (Thermo Fisher Scientific, San Jose, CA, USA), Then, lipidomics and metabolomics data were extracted using pre-processing within Workflow4Metabolomics® (W4M) (http://workflow4metabolomics.org) for automatic integration for each detected features (ions of given mass-to charge ratio and retention time) combined with CAMERA®, for annotation of isotopes and adducts and for normalization of intra- and inter-batch effects using QC samples. The resulting features [m/z; RT]) for each sample was subsequently manually sorted out according to their quality of integration and filtered by a 30% relative SD cutoff within the repeated pooled QC injections. Thereafter, accurate mass measurement of each putative metabolite was submitted to LIPID Metabolites And Pathways Strategy (LipidMaps®, www.lipidmaps.org), Human Metabolite Data Base (HMDB®, www.hmdb.ca), Biofluid Metabolites Database (MetLin®, metlin.scripps.edu) and milk metabolome database (MCDB®, www.mcdb.ca) annotation. The GlycoWorkBench software was used to draw HMOs structures. Moreover, the use of all ion fragmentation, when reverse phase chromatography was applied, helped us identify the proposed lipids, metabolites by examination of the (pseudo) tandem mass spectrum generated, combined with the use of in-house reference databanks (Ferchaud-Roucher et al., 2015; Courant et al., 2012). Metabolite's identification level was level one, for metabolites definitively annotated with our home data base (i.e., based upon characteristic physicochemical properties of a chemical reference standard (m/z,RT) in our in-house reference databanks and their M/MS spectra compared to those of breastmilk QC) or level two, for metabolites putatively annotated (i.e., without chemical reference standards, based upon physicochemical properties and MS/MS spectral similarity with public/commercial spectral libraries, e.g.,LipidMaps®,MetLin®, and MCDB®).

2.4. Breast milk targeted free amino acids (FAAs) and total fatty acids (FAs) analysis

FAA and FA concentrations were determined in expressed breast milk samples, collected at week 2 and week 4 of lactation. FAAs were separated and quantified, using Acquity H-Class® Ultra Perfomance Liquid Chromatography system (Waters Corporation, Milford, CO, USA)

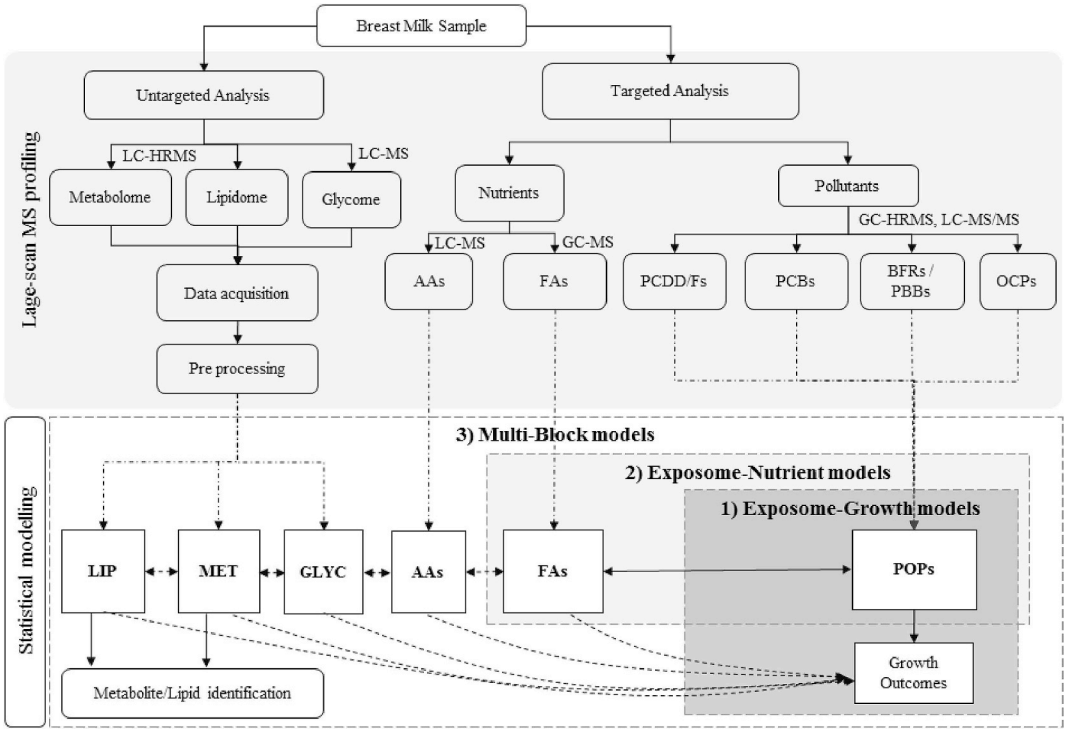


Fig. 2. Analytical workflow for the targeted and untargeted breastmilk profiling and statistical analysis for the molecular profiling and biomarkers identification. Abbreviations: AA, amino acids; BFR, brominated flame retardants; FAs, fatty acids; GC, gas chromatography; GLYC, glycome; HRMS, high resolution mass spectrometry; LC, liquid chromatography; LIP, lipidome; MET, metabolome; MS, mass spectrometry; OCP, organochlorinated pesticides; PBBs, polybrominated biphenyls; PCBs, polychlorinated biphenyls; PCDD/Fs, polychlorinated dibenzodioxins and dibenzofurans.

combined to a Xevo TQD® mass spectrometer (Waters Corporation, Milford, COMA, USA), following derivatization using AccQ®TagTM Ultra reagent (Waters Corporation, Milford, USA), as previously described (Alexandre-Gouabau et al., 2019). FAs concentrations were determined using the modified liquid–liquid extraction method of Bligh-Dyer and were analysed by gas chromatography using an Agilent Technologies 7890A® instrument (PerkinElmer, France), following trans-esterification previously described (Alexandre-Gouabau et al., 2018).

2.5. Breast-milk targeted profiling of persistent organic pollutants

The methodologies applied to isolate, detect, and quantify the targeted POPs including dioxins (17 PCDD/F congeners), polychlorobiphenyls (PCB) (12 dioxin-like and 6 non-dioxin-like PCB congeners), polybromodiphenylethers (8 PBDE congeners), polybromo biphenyls (3 PBB congeners) and organochlorine pesticides (30 OC compounds) have been previously described (Antignac et al., 2009; Costera et al., 2006; Cariou et al., 2005; Antignac et al., 2009; Bichon et al., 2015). Briefly, ^{13}C-labelled congeners were added to each sample for quantification according to the isotopic dilution method. Breast milk samples were first submitted to a liquid/liquid extraction with pentane. Resulting extracts were weighed to measure fat content (gravimetric method) and reconstituted in hexane for further sample clean up. Gel lipid compounds originated from the matrix. For other targeted substances, three successive acid silica, florisil, and Celite/carbon columns were applied for lipid removal, fractionation and further purification, respectively. PCDD/F, PCB, PBDE and OC measurements were performed by gas chromatography (Agilent 7890 A) coupled to high-resolution mass spectrometry (GC-HRMS) on electromagnetic sector instruments (JEOL MS 700D or 800D®), operating at 10,000 resolution and in the single ion monitoring (SIM) acquisition mode. Hexabromocyclododecane (HBCD) isomers were quantified using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on a triple quadrupole instrument (Agilent 6410®). All these methods were fully validated according to current European criteria in the field of regular control of foodstuff of animal origin and accredited according to the ISO 17,025 standard. Quality assurance/control (QA/QC) procedures included systematic analysis of negative and positive control samples in each batch of analysed samples. Recoveries were classically calculated according to the rules defined at regulatory level (Commission Regulation 2012) and were in the 80–120% range. The method's extended uncertainty was lower than 20%. In order to investigate not only contamination levels but also contamination patterns, all determined POPs concentrations were secondly expressed as relative contributions (%) of each particular congener to the sum of all congeners from the same family.

2.6. Postnatal POP exposure estimation

The infant *i* postnatal exposure to the contaminant *n* ($E_{T2,n,i}$, ng/kg bw/day) from breast milk, was estimated by the combination of the individual normalised breastmilk intake (INT_i, mL/kg bw/day), the lipid normalised concentration in breastmilk sample *p* of contaminant *n* (*POPn*; ng/g lipids) and the specific content of lipids in breastmilk sample *p* (LIP; g lipids/100 mL milk) as follows:

$$E_{T2,i,n} = INT_i \; x \; POP_{p,n} \; x \; LIP_p$$

This approach is analogous to the estimation of the estimated daily intakes (EDI) used by WHO and allows the comparison with the safety values like the tolerable daily intakes (TDI) for given contaminants (WHO, 2000). These estimates only consider the exposure from human milk, not eventual contamination of formulas, that would be expected to be similar for all infants.

2.7. Statistical analysis

Individual characteristics were summarized with median and interquartile range, for continuous variables, and frequency and

percentage, for categorical variables. The tertiles of delta weight Z-score (difference between weight Z-score at birth and hospital discharge) was used to rank the infants in 3 groups: “Slower growth”, “Medium growth”; “Faster growth”. The statistical comparison of individual characteristics between groups (i.e. faster, medium and lower growth) was performed using Kruskal Wallis Test and Fisher's exact test, for numerical and categorical variables, respectively. The distributions of POPs among groups were summarized by medians and interquartile ranges and compared statistically as continuous variables. Those chemicals with detection rates below 75% were excluded from the statistical analysis. For four analytes (PBDE 28, 154, 183 and 209) whose detection rates were between 93 and 98%, the non-detected values were replaced by limits of detection (Antweiler, 2015).

The bivariate associations between milk POPs and fatty acid variables were calculated with Spearman's correlation coefficients (ρ) and plotted them in heatmaps. Furthermore, sparse partial least squares (sPLS) was conducted to identify association patterns between milk POPs and nutrients (FAs and macronutrients) considering the correlation structure of both datasets, and displayed by means of network plots (Chun and Keleş, 2010). The association between the infant exposure to POPs (pg or ng/Kg BW/day, log-scale) and infant growth during hospital stay (Delta Z-score) was estimated through multivariate linear regression considering known confounding variables such as maternal age, maternal body mass index (Kg/m^2, log-scale), birth weight (grams, log-scale), gestational age (weeks), newborn sex, hospital stay duration (days) and energy intake (Kcal/kg/day, log-scale) from complementary parenteral and enteral nutrition. The two-way product interaction terms between considered variables were evaluated for statistical significance. The associations are summarized using the beta regression coefficients and 95% confidence intervals.

For a sub-group of 19 infants with duplicated breast milk sample measurements (Week 2 and Week 4, n = 38) of lipidome (LIP), metabolome (MET), glycome (GLY), amino acids (AAs) and fatty acids (FAs), we extended the analytical framework towards a more comprehensive approach to integrate the chemical complexity of breastmilk in the growth models. Assuming the computational limitations associated to the minimal sample size of this sub-group, in this section we mainly aimed to set-up and evaluate the applicability of a large-scan spectrometric platform coupled to multi-block models for a more comprehensive exploration. The results associated to the nutritional fraction has been published elsewhere, thus in this publication we will emphasize the interplay between POPs and the different nutritional fractions (Alexandre-Gouabau et al., 2018, 2019). In this regard, we finally integrated the six pre-processed (normalised and standardized) data-blocks from targeted methods, including POPs (n = 52 variables), FAs (n = 34 variables), AAs (n = 16 variables), and the matrices from non-targeted methods, GLY (n = 75 variables), LIP (n = 488 variables) and MET (n = 221 variables) against the infant growth (weight delta Z-score between birth and discharge) dichotomized to generate two groups of infants (“faster growth” and “slower growth”). The group of “medium” growth was not considered in this pilot study favouring the identification of molecular signatures discriminating the more extreme growth trajectories. The MS-based metabolomics and lipidomic features were previously submitted to a throughout pre-processing and filtering process of raw spectral data using Analysis of Variance- Partial Least Square (AoV-PLS) (Ghaziri et al., 2015) to reduce the internal redundancy and to pintpoint the most discriminant polar and apolar features providing a clear separation between the two infant growth groups, obtaining the final sets of 488 lipidomic and 211 metabolomic variables (Alexandre-Gouabau et al., 2018, 2019). The datasets were integrated using the sparse generalized canonical correlation models (Tenenhaus and Tenenhaus, 2014) extended to a discriminant mode so-called DIABLO (Data Integration Analysis for Biomarker discovery using Latent variable approaches for ‘Omics studies) workflow implemented with the MixOmics R package (Rohart et al., 2017; Singh et al., 2019). Briefly, similar to the partial least-squares regression, the model projects latent variables (i.e. components) based on linear combinations of underlying features maximally correlated across data-blocks and the response variable, performing variable selection via L1 penalisation (Tibshirani, 2011) to support the data-driven variable selection. The main parameters of the model are the correlation matrix design that pre-establish the degree of association known between blocks (between 0 and 1), number of components and degree of penalisation (number of variables to retain) for each component and block. The number of components is optimized through another 10-fold cross-validation (CV) process seeking at minimizing the misclassification model error rate whereas the degree of penalisation is also driven by an internal CV procedure. The statistical analysis was conducted entirely using the R software, v. 3.5.0, with the MixOmics R package.

3. Results

3.1. Levels of contaminants in breast-milk and infant post-natal exposure estimates

The concentrations of POPs are summarized in Supplemental Table 2 expressed in lipid basis, confirming the presence of all monitored POPs in the analysed breastmilk samples. Mean dioxin toxic equivalent factors (TEF) of 6.17 and 4.50 WHO TEF_{2005}/g l. w. were observed for the sum of PCDD/Fs and dioxin-like PCBs, respectively. The mean sum concentrations of non-dioxin like PCBs, PBDEs and PBB153 resulted in respective values of 87.27, 1.7 and 0.08 ng/g l. w. Strong positive Spearman's rank bivariate correlations were noticed between POPs from the same family, whereas weaker correlations were noticed between family comparisons, especially for the case of PBDEs (Fig. S1).

3.2. Levels of macronutrients, fatty acids and correlations with POPs

The summary of levels for macronutrients and FAs in breastmilk can be found in Supplemental Table 3. The correlation analysis of targeted lipophilic chemicals in breastmilk revealed strong positive associations between ω3 PUFAs (docohexaenoic acic or DHA, docosapentaenoic acid or DPA and eicosapentaenoic acid or EPA) and most POPs, most substantially for TCDD, non-coplanar and non-dioxin like PCBs (Fig. 3). These toxicants were also strongly correlated to saturated FAs such as pentadecanoic acid (15:0), palmitic acid (16:0), stearic acid (18:0) and arachidic acid (20:0). Some strong inverse correlations were also noticed for ω6 PUFAs and non-coplanar PCBs, and specially docosapentaenoic acid ($22:5_{n\text{-}6}$), docosadienoic acid ($22:2_{n\text{-}6}$) and linoleic acid ($18:2_{n\text{-}6}$) exhibiting the strongest negative correlation with most POPs. The bivariate correlation analysis was extended with sPLS allowing the efficient integration of two correlated matrices and the identification of the most relevant variables and their networks, displayed in Fig. 4. The relevance networks display the topological associations between FAs and POPs considering a cut-off level of bivariate correlation of 0.34 to favour the visualization of most relevant associations. This approach showed that FAs, 15:0 (pentadecanoic acid) and 20:1 (eicosenoic acid) appeared as central nodes on the association network with PBDEs, highlighting the strong negative associations between 15:0 and PBDE99 and positive associations between 20:1 and PBDE99 or the sum of PBDEs. Another network was built around total lipids and energy, negatively associated to PCBs and around the saturated FAs 20.0 (arachidic acid) and 18.0 (stearic acid) displaying positive associations with coplanar PCBs such as PCB114.

3.3. Associations between POPs and preterm infant growth

First, we explored the distribution of estimated daily intake of POPs through maternal breastmilk intake during the hospital stay for the infant growth groups (“Slower”, “Medium” and “Faster” growth) established by the tertiles distributions of weight z-score difference between birth and hospital discharge (weight Delta z-score). The Estimated Dietary Intake (EDI) of POPs for the different Delta z-score are summarized in Table 1, showing higher concentration levels of several POPs among the “Faster” group, especially dioxin-like PCBs (e.g. PCB 126, 127, 157). In order to

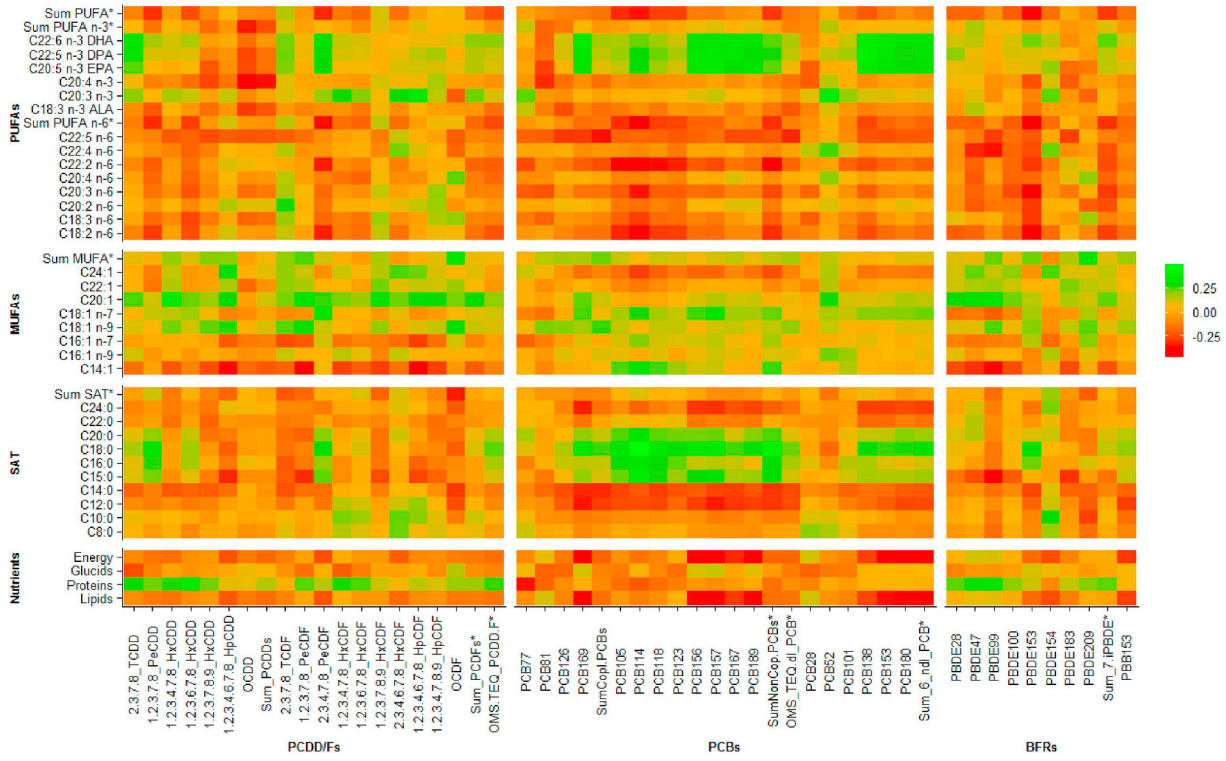


Fig. 3. Heatmap representing the bivariate correlations between macronutrients, saturated (SAT), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids with persistent organic pollutants in breast-milk. The Spearman correlation coefficients are represented by the colour scale. The grouped variables as sum of individual concentrations are identified by an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

account for known confounding variables related to infant growth, we further conducted multivariate linear regression considering maternal age, BMI, birth weight, hospitalisation days and energy intake as covariates. The regression coefficients (and 95% confidence intervals) for the different pollutants and models are displayed with forest-plots in Fig. 5, showing the observed associations between the different POPs and infant growth. Overall, the results showed the strong confounding effect of energy intake underlying the positive association between the

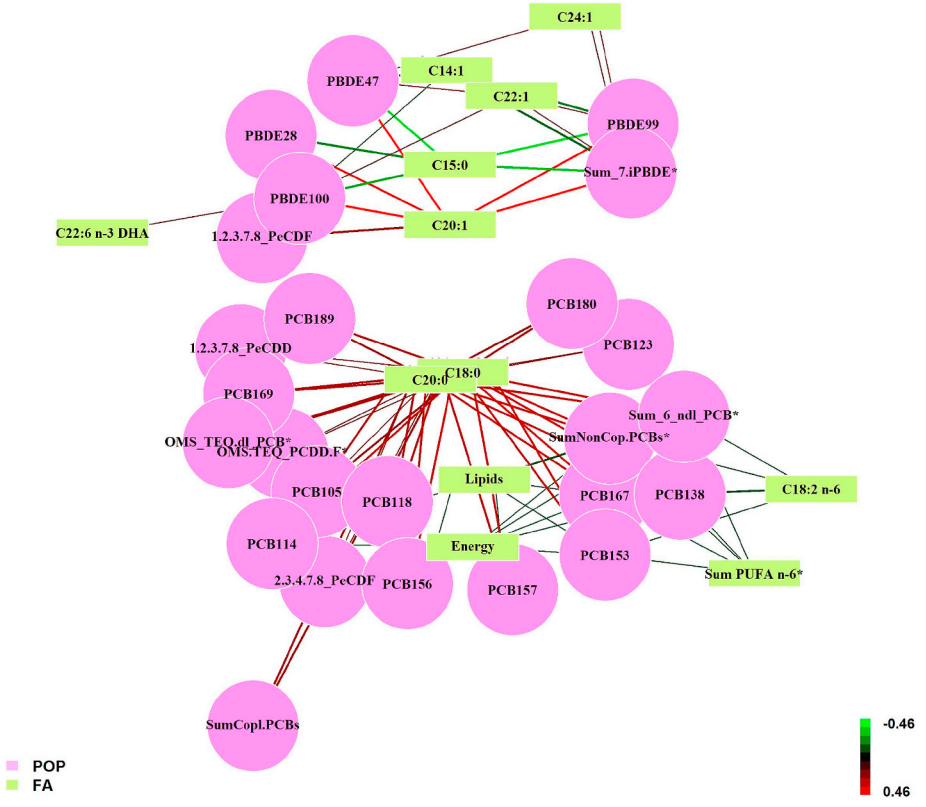


Fig. 4. Relevance network plots representing the strongest associations between fatty acids (green boxes) and pollutants (purple boxes) using sparse partial least squares. The strength of the correlations between pollutants and fatty acids is represented by colour key of edges. The threshold of correlations was set up at −0.28 (negative associations, black-green) and 0.28 (positive associations, red) in order to highlight the most relevant clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Summary of estimated dietary intake (Median (Interquartile range) of persistent organic pollutants, macronutrients and energy from breast milk for preterm infants with slower, medium and faster growth trajectories. Infants were grouped in tertiles of weight z-scores difference between birth and discharge. P-value was estimated using Mann-Whitney-Wilcoxon Test.

	Slower N = 23	Medium N = 22	Faster N = 23	P-value
Macronutrients and energy daily intake				
Energy enteral total [a]	123.7 (79.1–130.4)	104.4 (83.6–133.6)	124.3 (111.3–135.1)	0.29
Proteins enteral total [b]	2.5 (1.4–2.7)	1.8 (1.3–2.6)	2.4 (1.9–2.7)	0.21
Lipids enteral total [b]	5.2 (3.7–6.5)	5.7 (3.5–7.1)	5.6 (5.0–7.5)	0.31
Carbohydrates enteral total [b]	14.3 (8.8–15.6)	10.2 (7.9–13.6)	14.8 (12.2–15.4)	0.10
Energy breast-milk [a]	91.0 (72.1–102.9)	99.7 (77.8–118.6)	107.3 (101.2–112.1)	0.07
Proteins breast-milk [b]	1.3 (1.1–1.7)	1.4 (1.0–1.7)	1.6 (1.4–1.7)	0.09
Lipids breast-milk [b]	4.5 (3.7–5.3)	5.7 (3.3–7.1)	5.6 (5.0–7.1)	0.09
Carbohydrates breast-milk [b]	10.8 (7.1–11.7)	10.2 (6.5–11.9)	11.8 (9.7–12.3)	0.08
Persistent organic pollutants daily exposure				
2.3.7.8-TCDD [c]	1.8 (1.3–3.3)	2.0 (1.3–2.7)	2.7 (1.6–4.6)	0.07
1.2.3.7.8-PeCDD [c]	6.4 (4.2–11.0)	7.5 (5.4–9.1)	11.5 (5.8–14.0)	0.10
1.2.3.4.7.8-HxCDD [c]	2.8 (2.0–4.7)	2.8 (2.3–3.8)	4.2 (2.9–5.5)	0.06
1.2.3.6.7.8-HxCDD [c]	15.6 (10.3–25.2)	18.2 (13.4–23.9)	27.7 (14.2–37.8)	0.16
1.2.3.7.8.9-HxCDD [c]	3.9 (2.4–5.3)	3.7 (2.6–4.5)	5.2 (3.7–8.0)	0.09
1.2.3.4.6.7.8-HpCDD [c]	17.4 (12.4–30.1)	17.8 (13.2–25.4)	23.4 (16.8–31.5)	0.30
OCDD [c]	93.1 (59.8–141.6)	89.6 (66.3–141.0)	105.9 (79.5–141.5)	0.62
Sum PCDDs [c]	157.1 (93.4–241.1)	136.9 (109.8–221.5)	167.1 (149.0–238.1)	0.33
2.3.7.8-TCDF [c]	1.2 (0.6–2.1)	1.3 (1.0–1.6)	1.8 (1.1–2.5)	0.19
1.2.3.7.8-PeCDF [c]	0.7 (0.4–1.3)	0.9 (0.7–1.0)	1.1 (0.6–1.4)	0.26
2.3.4.7.8-PeCDF [c]	15.1 (8.8–26.0)	17.8 (10.7–20.8)	21.6 (13.2–37.5)	0.13
1.2.3.4.7.8-HxCDF [c]	4.6 (3.4–7.1)	5.6 (3.9–7.2)	5.6 (4.3–9.7)	0.25
1.2.3.6.7.8-HxCDF [c]	4.6 (3.2–7.0)	5.9 (3.7–6.8)	6.4 (4.3–9.9)	0.23
1.2.3.7.8.9-HxCDF [c]	0.3 (0.1–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.5)	0.28
2.3.4.6.7.8- HxCDF [c]	2.5 (1.5–3.8)	2.5 (1.7–3.3)	2.6 (1.7–4.2)	0.50
1.2.3.4.6.7.8-HpCDF [c]	4.0 (2.3–5.1)	3.9 (3.4–6.1)	4.0 (2.8–6.2)	0.70
1.2.3.4.7.8.9-HpCDF [c]	0.3 (0.3–0.6)	0.4 (0.3–0.6)	0.4 (0.3–0.5)	0.53
OCDF [c]	0.8 (0.6–1.2)	0.9 (0.5–1.5)	1.0 (0.9–1.6)	0.17
Sum PCDFs [c]	35.0 (20.9–54.9)	41.0 (32.1–49.2)	48.0 (31.1–70.4)	0.19
OMS-TEQ PCDD/F [d]	15.6 (11.4–27.6)	19.0 (13.9–22.7)	25.7 (14.9–36.5)	0.12
PCB 77 [c]	9.4 (7.4–14.0)	11.7 (7.7–19.5)	14.5 (9.4–23.4)	0.13
PCB 81 [c]	5.3 (3.0–9.4)	4.8 (3.0–7.1)	7.4 (4.7–12.3)	0.31
PCB 126 [c]	77.5 (40.5–174.4)	83.2 (50.7–146.6)	123.2 (100.8–314.9)	0.04
PCB 169 [c]	51.6 (28.4–99.8)	49.3 (34.6–58.8)	88.2 (43.1–136.6)	0.06
Sum Copl. PCBs [c]	142.2 (78.3–317.2)	148.3 (106.1–243.0)	222.7 (170.6–502.9)	0.04
PCB 105 [c]	5001.6 (2434.2–10,041.8)	5385.0 (3028.2–9069.6)	8058.6 (6816.1–14,580.4)	0.05
PCB 114 [c]	1177.5 (481.4–2158.6)	950.8 (674.7–1549.6)	1892.2 (1116.4–3229.9)	0.04
PCB 118 [c]	22,777.3 (10,346.1–44,823.8)	20,028.5 (12,433.6–35,803.7)	38,553.2 (27,118.5–71,359.0)	0.04
PCB 123 [c]	240.5 (119.0–524.5)	241.4 (119.1–468.7)	444.9 (334.8–842.8)	0.03
PCB 156 [c]	9208.5 (4182.1–18,217.1)	8799.5 (5088.2–10,683.7)	15,913.1 (7700.4–20,898.5)	0.06
PCB 157 [c]	1743.5 (687.5–3653.1)	1353.4 (835.1–1844.1)	2923.9 (1329.0–3435.5)	0.03
PCB 167 [c]	2422.3 (1117.8–6153.4)	2203.1 (1611.9–3375.9)	4658.6 (2565.7–6980.3)	0.03
PCB 189 [c]	873.4 (408.8–1790.9)	786.8 (539.5–1000.3)	1855.5 (665.4–2200.5)	0.08
Sum Non Cop. PCBs [c]	42,336.3 (19,966.5–90,500.2)	36,028.2 (25,865.5–65,409.0)	77,086.9 (48,508.3–144,809.2)	0.03
OMS-TEQ dl-PCB [d]	10.0 (5.2–24.2)	10.3 (7.2–18.6)	15.7 (13.7–41.0)	0.04
PCB 28 [e]	3.8 (2.4–6.6)	3.7 (2.1–8.0)	5.0 (2.9–7.5)	0.69
PCB 52 [e]	0.8 (0.5–1.0)	0.8 (0.5–1.4)	0.9 (0.7–1.5)	0.44
PCB 101 [e]	1.3 (0.8–1.7)	1.2 (0.8–2.2)	1.9 (1.4–2.1)	0.10
PCB 138 [e]	50.5 (23.3–121.9)	59.6 (36.9–82.4)	97.9 (56.2–145.1)	0.05
PCB 153 [e]	107.2 (47.4–229.1)	111.6 (74.0–141.7)	197.8 (97.7–256.8)	0.10
PCB 180 [e]	60.4 (29.3–125.6)	61.6 (39.7–70.4)	124.5 (47.2–184.2)	0.06
Sum 6 ndl-PCB [e]	221.5 (100.0–484.3)	231.1 (162.5–313.3)	456.1 (203.2–578.0)	0.07
PBDE 28 [e]	0.11 (0.05–0.15)	0.09 (0.06–0.15)	0.11 (0.08–0.16)	0.56
PBDE 47 [e]	1.30 (0.64–1.79)	1.27 (0.77–2.23)	1.22 (0.88–2.54)	0.48
PBDE 99 [e]	0.24 (0.15–0.40)	0.27 (0.17–0.40)	0.24 (0.20–0.48)	0.60
PBDE 100 [e]	0.34 (0.14–0.52)	0.34 (0.17–0.59)	0.34 (0.25–0.76)	0.50
PBDE 153 [e]	1.76 (0.95–2.65)	1.38 (0.95–2.62)	2.46 (1.73–3.03)	0.12
PBDE 154 [e]	0.05 (0.03–0.09)	0.08 (0.05–0.19)	0.07 (0.04–0.09)	0.18
PBDE 183 [e]	0.14 (0.09–0.23)	0.16 (0.10–0.22)	0.18 (0.14–0.30)	0.20
PBDE 209 [e]	0.87 (0.23–1.77)	0.52 (0.11–2.41)	0.40 (0.05–1.21)	0.25
Sum 7 i PBDE [e]	4.34 (2.17–5.57)	4.63 (2.45–7.34)	4.94 (3.88–6.76)	0.39
PBB 153 [e]	0.22 (0.11–0.46)	0.18 (0.14–0.23)	0.25 (0.17–0.66)	0.04

Units.
[a] Kcal/kg bw/day.
[b] g/kg bw/day.
[c] pg/kg bw/day.
[d] TEF/kg bw/day.
[e] ng/kg bw/day.

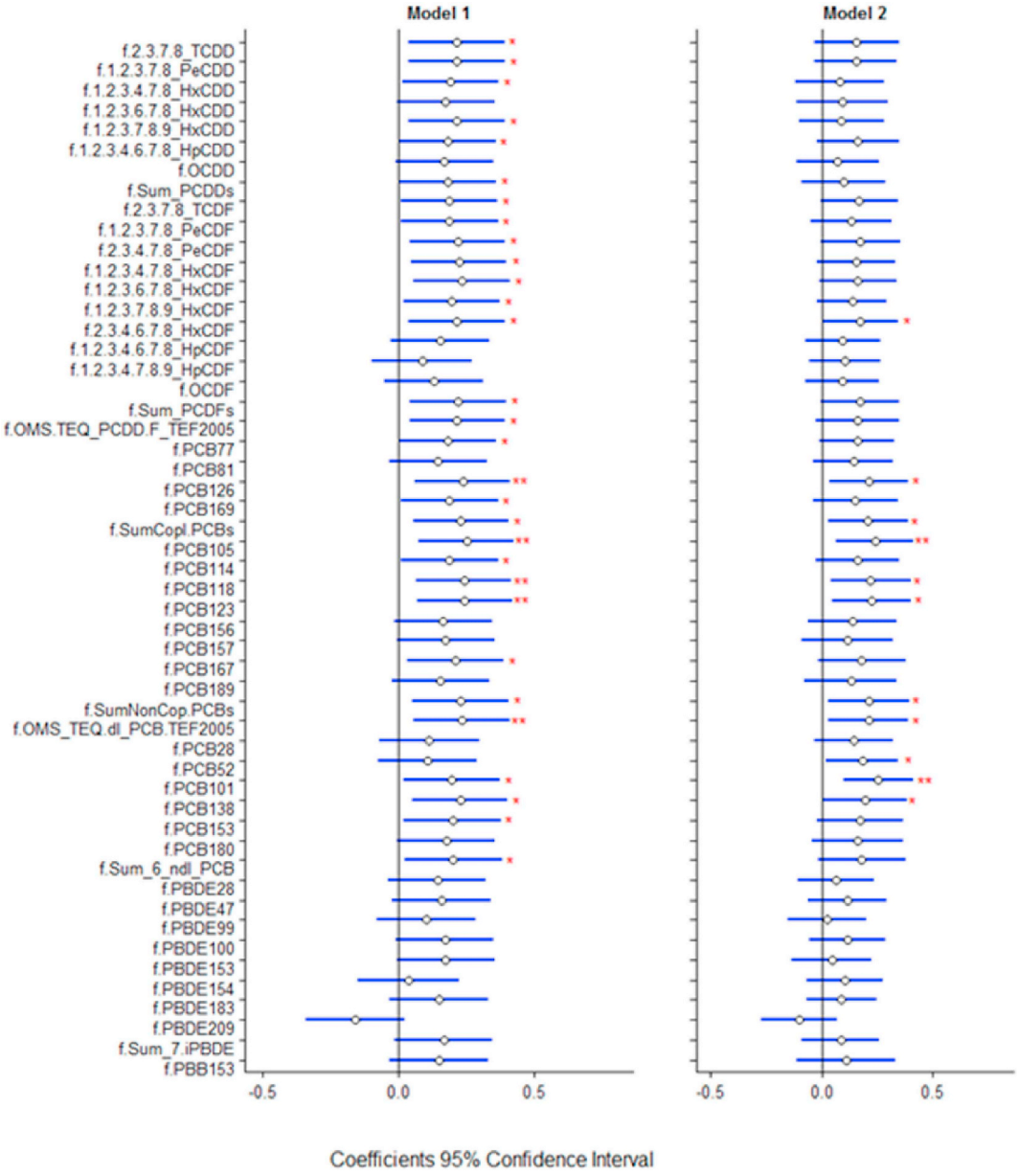


Fig. 5. Forest plot representing the associations (β coefficient, 95% confidence interval) between postnatal exposure of preterm infants to POPs (continuous variable, log-scale), through breastmilk, and growth during hospital stay, measured as the difference of weight Z-score between birth and discharge (continuous variable) (n = 68). **Model 1**, crude model. **Model 2**, adjusted for birth weight, hospital stay duration, maternal age, maternal body mass index and infant energy intake. Statistical significance is represented of coefficients is represented by * $p < 0.05$ and ** $p < 0.01$.

exposure to POPs and preterm infant growth during the hospital stay (Model 2). After the adjustment by energy intake, some chemicals exhibited positive significant associations (i.e. PCB101, 105, 118, 126, 138) with postnatal preterm infant growth. The EDI of POPs through breastfeeding was also modelled against the infant weight z-score at 24 months showing positive associations for a substantial number of dioxins after the adjustment for the confounding factors including, energy intake (Fig. 6). The larger associations were depicted by 2.3.4.7.8-PeCDF ($p = 0.009$) and the sum of PCDD/Fs WHO-TEQ ($p = 0.01$). Some furans such as 2.3.4.6.7.8-HxCDF were also found consistently associated with preterm infant growth throughout both growth time points.

3.4. *Integrated expotypes and nutritypes associated to preterm infant growth*

After the iterative tuning process conducted within DIABLO framework, we finally selected a null design for the correlation matrix that emphasized the selection process of most discriminatory variables and maximized the separation of growth groups. The CV optimization process led us to setup the model with 2 components. We established, for the blocks lipidome, metabolome, glycome, POPs, FAs and AAs, a list of 20, 7, 7, 9, 5 and 5 variables selected, respectively in the first component and 10, 7, 5, 7, 5 and 5 variables selected for the second component. The scatter plots from the multiblock model showed the most discriminant ability for the blocks lipidome, metabolome and POPs (Fig. S2). Among the unknown molecules retained from the non-targeted approaches, we were able to finally annotate 14 lipids, including five phosphatidylethanolamines (plasmalogen-derivative PE (38:5e): PE (O-18:0/20:5), PE (38:3): PE (18:0/20:3), PE (40:4): PE (20:4/20:0), PE (38:4): PE (18:0/20:4), PE (38:1): PE (20:0/18:1)), three phosphatidylcholines (PC(36:2): PC(18:0/18:2), PC(42:1): PC(18:1/24:0) and PC(30:1): PC(14:0/16:1)), two sphingomyelins (SM (d18:1/16:1) and SM(d18:1/24:0)), one diacylglycerol (22:5n-3/22:6n-3), one fatty acyl (3-hydroxy-adipic acid), one lysophosphatidylglycerol (LPG (22:4)), and one leukotriene (M10,11-dihydro-20-trihydroxy-

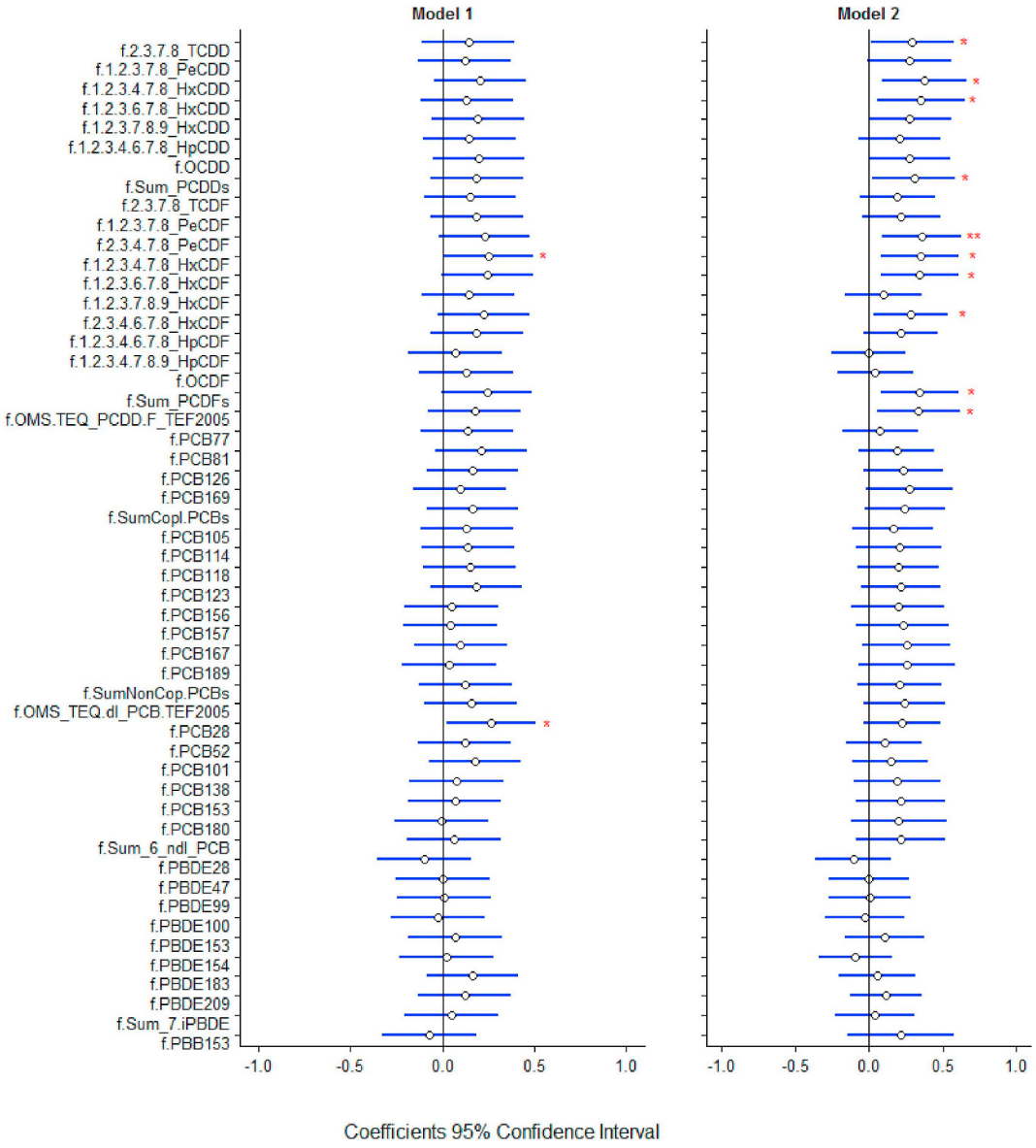
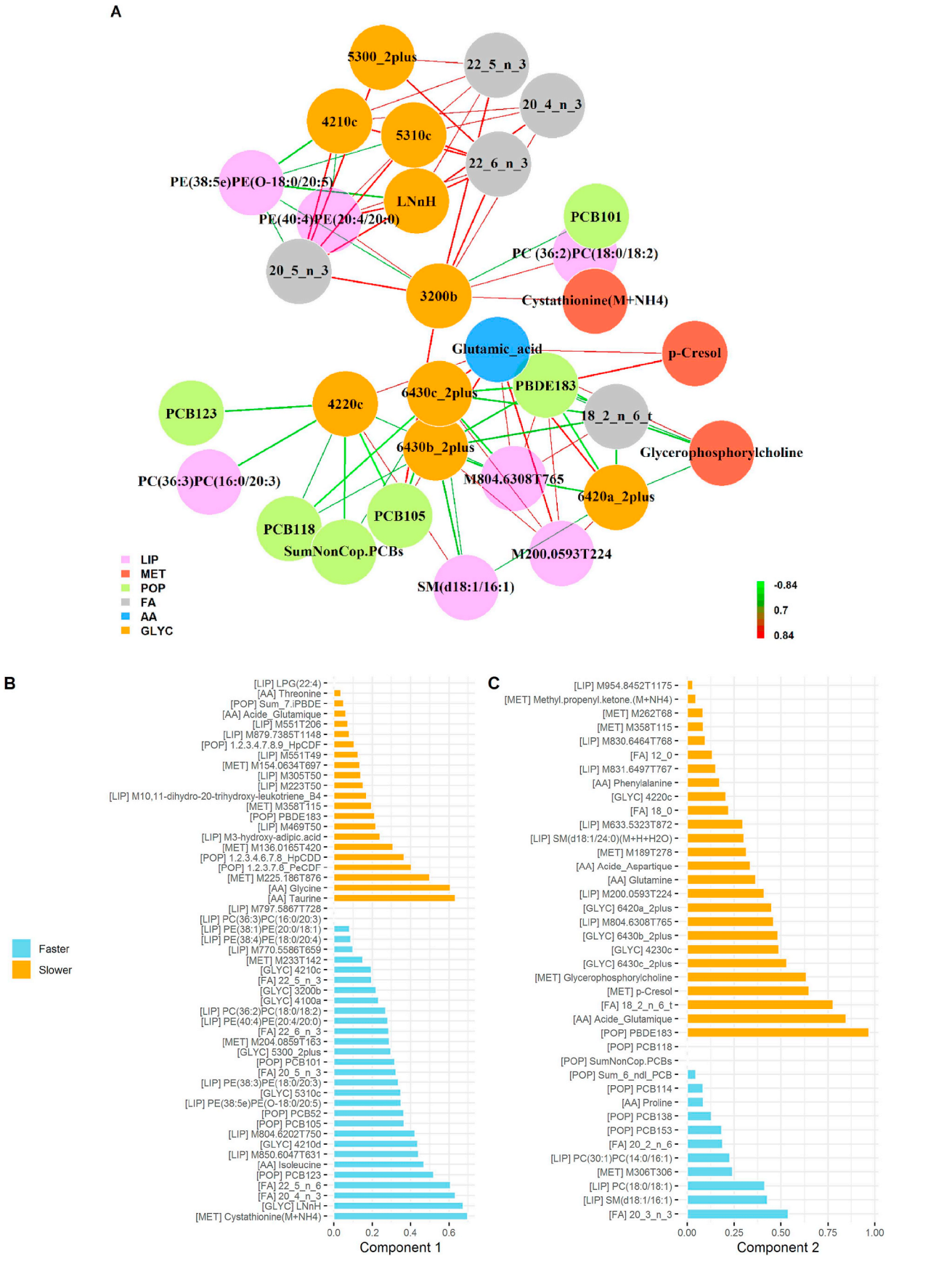


Fig. 6. Forest representing the associations (β coefficient, 95% confidence interval) between exposure of preterm infants to POPs (continuous variable, log-scale) through breastmilk and weight z-score at 24 months (continuous variable) (n = 54). **Model 1**, crude model. **Model 2**, adjusted for birth weight, infant energy intake during delivery hospital stay, maternal age and maternal BMI. Statistical significance of coefficients is represented by * $p < 0.05$ and ** $p < 0.01$.

leukotriene-B4). Additionally, we identified 3 molecules from the metabolome dataset (cystathionine, glycerophosphorylcholine and p-cresol). The associations between variables across blocks can be visualized in the correlation individual plots (Figs. S3A and S3B) and in the correlation between molecular variables in the circle plots (Figs. S3C and S3D) for the component 1 and 2, respectively. The circle plot from the component 1 highlighted mainly positive correlations between lipids with oligosaccharides and some POPs, conversely the component 2 showed a higher number of negative correlations between biomarkers, specially between glycome, lipidome datasets, some POPs and the glutamic acid. The relevance network plot displayed in Fig. 7A highlights the most relevant clusters of variables that discriminated the preterm infant growth groups, supported by the clustered image map (Fig. S4). The loading vectors for each selected variable from each molecular block is illustrated in Fig. S5, grouped by blocks, and in Fig. 7B and C, grouped by the growth class where the molecules are more abundant, sorted by the loading weights. Globally, the chemical signature observed for the "Faster growth" group exhibited a characteristic profile of polyunsaturated fatty acids type ω-3 and ω-6, phosphatidylethanolamines, phosphatidylcholines, the branched chain amino acid isoleucine, the oligosaccharide Lacto-N-neohexaose (LNnH) and the metabolite cystathionine. The "Slower growth" group was more associated with a molecular signature richer in the AAs: glycine, taurine and glutamic acid, the metabolite p-cresol, some saturated fatty acids, the hydroxyl adipic acid and a leukotriene. We also observed a different pattern among groups in terms of POPs exposure, being the PBDEs (specially the PBDE183 and some dioxins) more associated with the slower growth group, whereas the PCBs (i.e. 123, 105, 52) were more abundant in the faster growth group.

4. Discussion

The literature has previously shown that *in utero* exposure to some organochlorine pesticides including dichlorodiphenyldichloroethylene (*p,p'*-DDE) or chlordecone may be associated with preterm birth (Longnecker et al., 2001). The associations with other POPs, such as PCDD/Fs or PCBs were reported as insignificant (Casas et al., 2015; Ferguson and Chin, 2017; Longnecker et al., 2005). Nonetheless, one



(caption on next page)

Fig. 7. 7.A. Relevance network plot representing the group of molecular variables more associated with the growth of preterm infants (cut-off of absolute correlation set-up at ± 0.7) generated by the multiblock model. The colour code of circles identifies the different bocks: metabolome (red), purple (lipidome), glycome (orange), amino acids (blue), fatty acids (grey) and persistent organic pollutants (green). 7.B. and 7.C. Bar plots representing the loading vectors from both components generated by the multiblock model, grouped by the growth class ("Faster" in blue; "Slower" in orange) where the molecules were more abundant. The block identifiers can be found in brackets: amino acids [AA], fatty acids [FA], glycome [GLYC], lipidome [LIP], metabolome [MET] and persistent organic pollutants [POP]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

study has shown significant positive associations between exposure to PBDEs and the risk preterm birth (Peltier et al., 2015). To the best of our knowledge, no studies have explored the impact of postnatal exposure to POPs on early growth of preterm infants, highlighting the value of our small cohort. The nutritional interventions with fortified breastmilk are recommended to favour the high energy and protein intake required to achieve the normal postnatal growth and neurodevelopment of preterm infant (Gibertoni et al., 2015). Considering the internal exposure bio-amplification among low and very low birth weight infants, we hypothesized that the xenobiotic component of breastmilk, specifically lipophilic pollutants, could contribute to impair the normal preterm growth interacting with the nutritional fraction.

To this end, we have applied for the first time a novel comprehensive approach based on MS multi-platform coupled to an advanced data workflow to better understand the molecular complexity of breastmilk and its simultaneous impact on preterm infant growth. The analytical approach includes a combination of ultra-trace targeted methods to quantify accurately low abundance contaminants (i.e. dioxins) combined with global metabolomics and lipidomics profiling methods to extend the framework to capture potential nutrient-contaminant associations. The potential of metabolomics in epidemiological exposome-health studies is vast. Some examples include the identification of endogenous metabolites to gain insights into the underlying pathological processes or to better understand the role of exposure biomarkers and molecular mediators, informing about specific exposure-to-disease pathways (Assi et al., 2015; Chadeau-Hyam et al., 2011; Niedzwiecki et al., 2019). Additionally, the development of non-targeted metabolomics to identify exogenous stressors in human samples at a massive scale is growing rapidly (Andra et al., 2017; Dennis et al., 2017; Jones, 2016; Warth et al., 2017), shaping a novel path for environmental epidemiology. Actually, the high-throughput generation of exposure biomarkers has been already demonstrated as a promising tool to support data-driven approaches, however, a large sample size is imperative to statistically identify the subtle associations commonly found between environmental exposures and diseases with enough statistical power after correcting for the false discovery rate (Chung et al., 2019; Manrai et al., 2017). Currently, there is not consensus about the best approach (i.e. data-driven vs hypothesis-driven) to identify complex associations between chemical mixtures and health outcomes. On the one hand, data-driven or comprehensive approaches benefit of a high discovery potential by screening a large panel of molecules at the cost of correction for the false discovery rates. On the other hand, hypothesis-driven approaches takes advantage of available knowledge to depict more biologically consistent models. For that reason, we believe that combining ultra-trace methods with semi-target metabolomics may provide a technical compromise between accuracy and selectivity to extend hypothesis-driven approaches towards a more comprehensive paradigm.

The results from the linear models, built on 58 mother-infant dyads, between the postnatal exposure to POPs and growth outcomes did not support an impairment of preterm infant growth during the hospital stay (Delta weight z-score between birth and discharge) or at 24 months (weight z-score). Conversely, we observed statistically significant and positive associations between some PCBs and delta weight z-score strongly influenced by the adjustment by energy intake. Similarly, positive associations were observed between some dioxins and the weight z-score at 24 months. The associations were no longer significant after the FDR correction. The positive associations could be strongly confounded by the energy and fat intake of infants considering the limitations to accurately estimate the energy and milk intake among breastfeeding new-borns, especially during the time after the hospital stay. The lack of concordance between the growth models at discharge and 24 months could be explained by the limited information on infants' dietary habits and lifestyle potentially confounding the associations. Another hypothesis could be the obesogenic potential of some PCBs, for instance PCB-77 has been associated to increased adipogenesis and gain of body fat (Arsenescu et al., 2008). However, this was not supported by the results at 24 months, when the group of dioxins (inhibitors of adipogenic differentiation) positively associated with weight z-score (Hsu et al., 2010). Moreover, it is necessary to keep in mind that these expotypes and nutritypes signatures may be very different in case of full-term newborn infants. Indeed, preterm infants, particularly male infants, are reported to experience insufficient fat-free mass accretion but higher fat mass percentage during hospitalisation in neonatal intensive care unit (Simon et al., 2013) which likely interfere with lipophilic pollutants exposure. In any case, further research should extend the present study, especially with a larger population (preterm *versus* term), through a longer follow-up and gaining insight about the metabolic status and lean-fat mass ratio of children, taking into account their sex-related differences.

For the first time, we have shown with an integrative multi-platform approach the complex exposome-metabolome chemical space of human milk and its potential interactive impact on preterm outcomes. For instance, the milks from the 'slower' growth group presented a characteristic profile of saturated fatty acids whereas the 'faster' group were mainly polyunsaturated. The previous analysis of preterm milk reduced to the lipidomic block alone (Alexandre-Gouabau et al., 2018) highlighted the major effect of several ω-3 FAs, such as DHA and its precursors EPA and DPA on postnatal infant growth during the hospital stay. We also noticed high correlation between some PCBs and ω-3 FAs found in the analysed breastmilk, reflecting the common dietary pathways such as fish and seafood products. Interestingly, some studies have found that ω-3 FAs could counterbalance the harmful effects of POPs (Marushka et al., 2017). This interaction could also explain the inconsistent results on the beneficial effects of dietary intake of ω-3 FAs for cognitive function and growth of preterm infants that have been reported (Smith and Rouse, 2017). Interestingly, using the multiblock model we identified PBDE183 and some dioxins (12,378-PeCDF and 1,234,678-HpCDD), being more associated with the "slower" growth group. These chemicals were not highlighted by the linear growth models; thus, it may suggest that molecular covariation with the rest of molecules could mask an elusive underlying association. The impact of negative or positive confounding of nutrients in environmental epidemiology has been scarcely investigated, however the few studies that accounted for it has reported interesting findings. For instance the negative confounding was elucidated with methyl mercury (MetHg) and polyunsaturated fatty acids on the associations with neurotoxicity of children from Faroe Islands, well known by their high fish intake (Choi et al., 2014). The co-exposure of MetHg, omega-3 fatty acids and selenium has been reported also in cord blood, resulting in negative confounding and effect modification on the associations with foetal growth outcomes (Wells et al., 2016).

The higher nutritional content related with "faster" growth group is consistent with anabolic function associated to the specific identified nutrients and related metabolites, such as, for example, the higher abundance of isoleucine, a branched-chain amino acid, in the "faster" milks, an amino acid previously associated with a higher early weight gain (Alexandre-Gouabau et al., 2019; Kirchberg et al., 2015).

Conversely, as in the previous preterm milk metabolomics analysis without considering pollutants, we observed higher abundance of taurine and glycine, under their free form, in “slower” breast milk along with a lower total fat content. Taurine contributes to intestinal fat absorption, bile acid secretion and hepatic functions, but no associations have been reported with growth outcomes among preterm or low birth weight infants in randomized trials (Verner et al., 2007). The present results could be explained by a depleted bile acid conjugation by taurine and glycine in “slower” breast milk, that could subsequently limit the solubilisation of lipids and sterols in mixed micelles, resulting, in turn, in the limited uptake of lipids into enterocytes (Alexandre-Gouabau et al., 2019) in addition to preterm gastrointestinal immaturity. The extend of the impact of milk composition on infant's absorption of organochlorines remains largely unexplored, being most of pharmacokinetic models developed under very simplistic assumptions (Lehmann et al., 2014).

The nutritional metabolites identified in the present study were slightly different from our previous analysis without considering POPs, probably explained by multi-block approach used and the potential covariation due to the block of POPs (Alexandre-Gouabau et al., 2019). Actually, the development and application of multiblock models is in full emergence, especially in the field of molecular biology or clinical settings where the cross-omic and multi-way integration is more common (Sun and Hu, 2016; Wu et al., 2019). In the present study, we have applied a powerful statistical approach that allows the variable selection accounting for the correlation between variables but also between data-blocks, presumably outperforming the concatenation approaches (Singh et al., 2019). This proposed approach may extend the growing toolkit to analyse the complex exposome-health data structures, favouring the holistic analysis of multiple exposures and potential mediators (Lazarevic et al., 2019). Additionally, it could be easily coupled to generic physiologically based pharmacokinetic (PBPK) models for POPs to refine the exposure estimates through different exposure windows or to generate cumulative exposure signatures through the perinatal period (Verner et al., 2013).

5. Conclusions

To sum up, the results from the present study support the methodological potential of cross-platforms integration to elucidate complex exposome-health associations. Despite we applied the workflow to identify nutritional metabolites in human preterm breastmilk, this could be applied to other matrices such as serum or adipose tissue to identify endogenous metabolites favouring the functional phenotyping. Furthermore, these preliminary results did not elucidate an impairment of preterm infant growth associated to the postnatal exposure to POPs, supporting the use of human milk in the nutritional strategies for preterm newborns. However, further research will be required 1) to evaluate the associations with higher exposure concentrations of POPs and more complex mixtures with emerging contaminants, 2) to gain insight into the suggested nutrient-pollutants interactions and 3) to evaluate the potential obesogenic effect of POPs on preterm infants that could exacerbate their already acknowledged risk for metabolic diseases (Chehade et al., 2018).

Author contributions

C.-Y.B., J.-C.R.: conceived the LACTACOL study and design. C.-Y.B., B.L.B and J.P.A conceived the LACTAPOP study on a subset of samples. A.-L.R., Y.G., F.F., S.C.: conducted metabolomics analysis and/or metabolites identification. C.-Y.B., J.-C.R, H.B. managed the clinical data, including collection and data cleaning. M.-C.A.-G.: conceived and supervised the present metabolomics study conducted on a subset of infants of LACTACOL cohort; G.C.S and T.M. conceived the appropriate statistical tools and/or performed the statistical analysis; G.C.S conducted the statistical analysis and provided a first draft; M.-C.A.-G ; J. P.A, B.L.B, T.M., F.F., C.-Y.B. and D.D.: critically revised the manuscript for important intellectual content; and all authors: acquired, analyzed, or interpreted the data, and read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.109018.

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Titre : Une approche intégrée pour caractériser l'exposome chimique chez la femme : application à la recherche étiologique des troubles du métabolisme et de la reproduction

Mots clés : exposome chimique, métabolomique, obésité, endométriose, infertilité

Résumé : Les humains sont exposés à des mélanges de produits chimiques synthétiques tout au long de leur vie, connu comme “exposome chimique”. Les nouvelles avancées en spectrométrie de masse et de la science des données offrent des leviers pour mieux comprendre l'impact des produits chimiques sur la santé humaine. Ce document résume quelques applications de méthodes novatrices visées à mieux comprendre l'exposome chimique féminin et ses relations avec des troubles de santé, y compris l'altération de la croissance précoce, l'obésité infantile, l'endométriose, l'infertilité ou le cancer du sein. Des méthodes statistiques avancées sont utilisées pour mieux comprendre les associations complexes entre les mélanges chimiques et l'obésité, en mettant l'accent sur l'identification des interactions entre les nutriments et les polluants afin d'identifier potentielles stratégies préventives. Des modèles multipolluants sont également utilisés pour évaluer les liens entre mélanges de polluants organiques persistants et les issus de fécondation in vitro, l'endométriose et le cancer du sein. Des méthodes de métabolomique sont appliquées pour mieux comprendre les associations fonctionnelles entre les expositions chimiques et l'endométriose dans le cadre d'une étude pilote avec des modèles statistiques type multibloc. La section des perspectives expose les ambitions du candidat de poursuivre le développement et les applications afin de mieux comprendre l'exposome chimique en mettant l'accent sur ses impacts sur les troubles féminins. La dernière section du document présente le profil académique et de recherche du candidat, l'unité d'accueil et la capacité à mener les recherches proposées.

Title : An integrated approach to decipher the chemical exposome of women: application to etiological research of metabolic and reproductive disorders

Keywords : chemical exposome, metabolomics, obesity, endometriosis, infertility

Abstract : Humans are exposed to complex mixtures of synthetic chemicals during the entire lifespan, known as “chemical exposome”. The novel adances in mass spectrometry and data science, provides unique opportunities to better understand the impact of chemicals on human health. This document summarize some case studies on the application of novel methods to gain insight on the female chemical exposome and its relationships with disorders during the entire lifespan including altered early growth, childhood obesity, endometriosis, infertility or breast cancer. Advanced statistical methods are used to gain insight on complex associations between chemical mixtures and obesity, with special focus on the identification of nutrient-pollutant interactions to inform preventive strategies. Multipollutant models are alos used to assess the relationships between mixtrues of persistent organic pollutants and In Vitro fertility outcomes, endometriosis and breast cancer. Novel mass spectrometry approches for targeted metabolomic profiling are applied to gain insight on the functional associations between chemical exposures and endometriosis in a proof-of-concept study implementing integrative multiblock models. The section of perspectives elaborate the ambitions of the candidate to pursue further development and applications to better understand the chemical exposome with special focus on its impacts on female disorders. The last section of the document provides the candidate’s academic and research profile, the hosting unit and the capacity to conduct the proposed researches.