Universidade de Lisboa

Faculdade de Farmácia

The Multi-Variable Paradigm of the Risk of Exposure to Per- and Polyfluoroalkyl Substances (PFAS)

Bianca dos Santos Rocha

Trabalho de Campo orientado pela Professora Doutora Lucija Peterlin Mašič, Professora Catedrática, da Faculdade de Farmácia da Universidade de Liubliana, e coorientado pela Doutora Maša Kandušer, Investigadora Associada, da Faculdade de Farmácia da Universidade de Liubliana, e pela Professora Doutora Maria Henriques Lourenço Ribeiro, Professora Associada da Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

Substâncias perfluoroalquílicas e polifluoroalquílicas (PFAS) são uma classe complexa e em constante expansão de compostos químicos sintéticos, atualmente perfazendo um total de mais de 5000 compostos, omnipresentes no ambiente, na vida selvagem e nos seres humanos, estando a exposição aos PFAS atualmente associada a vários *outcomes* adversos em saúde e, consequentemente, a uma diversidade de quadros clínicos de doença. O seu potencial bioacumulativo e a sua toxicidade contribuem para uma representação alarmante do espetro de exposição, em todos os níveis tróficos, a uma escala global.

O presente estudo fornece uma análise exaustiva dos dados existentes - desde a contextualização em termos de grupo químico, a métodos de síntese e riscos toxicológicos ecológicos e subsequentes à exposição humana - ao mesmo tempo que contextualiza estes resultados, especialmente quando vistos em termos de possíveis "eventos nucleares" nas vias de imunossuscetibilidade e imunodeficiência. O trabalho experimental levado a cabo teve como objetivo estudar os efeitos de uma única exposição de 72 horas a quatro PFAS individuais, nomeadamente PFOA, PFNA, PFDA e PFUdA, através da exposição de THP-1, uma linha monócito-*like,* imortalizada espontaneamente, derivada do sangue periférico de um caso infantil de leucemia monocítica aguda. A viabilidade celular das células monocíticas foi determinada (através de um ensaio MTS), analisada e interpretada após exposição aos quatro compostos PFAS selecionados para estudo, a uma concentração que, embora ainda significativamente superior à verificada na maioria dos contextos de exposição real, é muito inferior à investigada na grande maioria dos estudos disponíveis, procurando explorar a lacuna no conhecimento existente relativamente aos leucócitos e particularmente, à exposição dos monócitos e à imunotoxicidade global.

Globalmente, este *Report* engloba uma análise detalhada e resumida dos riscos associados à exposição aos PFAS, abordando sempre que possível as atuais hipótese de mecanismo fisiopatológico, além de apresentar uma descrição completa e resultados do projeto de investigação desenvolvido, com o objetivo de avaliar a existência de uma possível ligação estrutura-resposta, realizando avaliações ao longo de todo o processo, onde, além disso, foi ainda observada uma alteração fenotípica significativa, possivelmente demonstrando um estado ativado (A-THP-1), especulando sobre possíveis etiologias mecanísticas, padronizando as condições experimentais para garantir a repetibilidade e otimizando os procedimentos e resultados do protocolo.

Palavras-Chave

Substâncias Per- e Polifluoroalquílicas (PFAS); Exposição; Risco; THP-1

Abstract

Per- and polyfluoroalkyl substances (PFAS) are a large, complex, and ever-expanding class of synthetic chemicals, currently almost prefacing over 5000 compounds, ubiquitous in the environment, wildlife, and humans, with PFAS exposure being now linked to several adverse health outcomes and, as a result, to a diversity of clinical patterns of disease. Their bioaccumulative potential and toxicity, contributes to an alarming picture of the exposure spectrum, across all trophic levels, on a global scale.

The present study provides a comprehensive analysis of existing evidence - covering from chemical group contextualization, synthesis methods to ecological and human-exposure-related toxicological hazards - while further contextualizing these findings, especially when viewed in terms of possible "core events" in the pathways of immunosusceptibility and impairment. The experimental laboratory work aimed to study the effects of a single 72-hour exposure to four individual PFAS, namely PFOA, PFNA, PFDA, and PFUdA, through exposure of THP-1, a spontaneously immortalized monocyte-like cell line, derived from the peripheral blood of a childhood case of acute monocytic leukemia. Monocyte viability upon exposure to the four test PFAS compounds, at a concentration which, although still significantly higher than the realistic average, is much lower than that investigated in the vast majority of the latest research available, was determined (through an MTS Assay), analyzed, and interpreted, addressing the existing knowledge gap regarding WBC, particularly, monocyte exposure and overall immunotoxicity.

Overall, this report encompasses, a detailed summarized analysis of the risks associated with exposure to PFAS, to a physio-pathological mechanism hypothesis scale, besides presenting a description and results of the research project developed aiming to evaluate a possible structure-response link, carrying out *throughout*-process evaluations, where furthermore, a significant phenotypic change has been observed, possibly demonstrating an activated state (A-THP-1), speculating on mechanistic insights, standardizing experimental conditions to ensure repeatability, and optimizing protocol procedures and outcomes.

Keywords

Per-/Poly-fluoroalkyl substances (PFAS); PFAS-exposure; PFAS health risk; THP-1 Cell Line

Abbreviations List

ALT	Alanine Aminotransferase
AST	Aspartate Aminotrasferase
ATCC	American Type Culture Collection
BBB	*Brain-Blood* Barrier
BMDL	Benchmark Dose Level
BMD	Benchmark Doses
CAR	Constitutive Androstane Receptor / Constitutive Activated Receptor
CNS	Central Nervous System
CRP	Reactive C-Protein
CVD	Cardiovascular Disease
DMSO	Dimethyl Sulfoxide
DPPC	Dipalmitoylphosphatidylcholine
ECF	Electrochemical Fluorination
ECHA	European Chemicals Agency
EDCs	Endocrine-Disrupting Chemicals
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ERα	Estrogen Receptor *alpha*
ETFE	Ethylene Tetrafluooethylene

ETO Etoposide

FBS Fetal Bovine Serum

FDA Food and Drug Administration

GGT *Gamma*-Glutamyl Transferase

GM Complete Growth Medium

HFPO-DA Hexafluoropropylene Oxide Dimer Acid

HPGA Hypothalamic-Pituitary-Gonadal Axis

HSCs Hematopoietic Stem Cells

IFN-γ Inteferon *Gamma*

LDL Low Density Lipoprotein

LPS Lipopolysaccharide

LynB Lymphocytes B

LynTc Lymphocytes $T_{citotoxic}$ / CD8

LynTh Lymphocytes T_{helper} / CD4

MoCRA Modernization of Cosmetics Regulation Act

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

NF-κB Nuclear Factor *kappa* B

Nrf2 Nuclear Factor Erythroid 2-Related Factor 2

NTP US National Toxicology Program

PBMCs Peripheral Blood Mononuclear Cells

PCBs Polychlorinated Biphenyls

PCPs Personal Care Products

PDT Population Doubling Time

PFAAs Perfluoroalkyl Acids

PFAS Per- and Polyfluoroalkyl Substances

PFB Perfluorobutane

PFBA Perfluorobutanoic Acid

PFBS Perfluorobutane Sulfonic Acid

PFCAs Perfluorocarboxylic Acids

PFCs Perfluorinated Compounds

PFDA Nonadecafluorodecanoic Acid

PFDoA Perfluorododecanoic Acid

PFDoDA Perfluorododecanoic Acid

PFHpA Perfluoroheptanoic Acid

PFHpS Perfluoroheptanane Sulfic Acid

PFHxA Undecafluorohexanoic Acid

PFHxS Perfluorohexane Sulfonic Acid

PFNA Perfluorononan-1-Oic Acid

PFOA Perfluorooctanoic Acid

PFOS Perfluorooctane Sulfonic Acid / Perfluorooctane Sulfonate

PFSAs Perfluoroalkane Sulfonic Acids

PFTOHs Fluorotelomer Alcohols

PFUdA Perfluoroundecanoic Acid

POPs Persistent Organic Pollutants

PPARs Peroxisome Proliferator-Activated Receptors

PPARα Peroxisome Proliferator-Activated Receptor *alpha*

PPARγ Peroxisome Proliferator-Activated Receptor *gamma*

PRRs Pattern Recognition Receptors

PTFE Polytetrafluoroethylene

RfD Reference Dose

RXR Retinoid X Receptor

SVHC Substances of Very High Concern

SXR Steroid and Xenobiotic Receptor

TB Trypan Blue

TDCs Thyroid Disrupting Chemicals

THs Thyroid Hormones

TNF-α Tumor Necrosis Factor *alpha*

TPSA Topological Polar Surface Area

USEPA United States Environmental Protection Agency

VLFU Vertical Laminar Flow Unit

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1 Introduction

Per- and Polyfluoroalkyl Substances (PFAS) are a large, complex, and ever-expanding class of synthetic chemicals, currently prefacing over 4730 compounds (1). Produced since the 1940s, with an impressively wide range of different physical and chemical properties, making them suitable for a broad range of consumer products and industrial applications. (2)

The origins of PFAS date to DuPont's chemical research, in 1938, on stable fluorinated refrigerants, where researchers ended up discovering, unintentionally, polytetrafluoroethylene (PTFE). Attention was turned to its corrosion-resistant properties, which eventually culminated in its use in valves in the first nuclear bomb.

After the first war, in 1947, further research and development efforts led to the widespread use of *Teflon*, trademark name for PTFE, possibly the most well-known PFAS, and one that remains in production today (2). Fast-forward to 2009, when DuPont began the commercial development of *GenX*, trademark name for *short-chain* organofluorine chemical compound, is the ammonium salt of hexafluoropropylene oxide dimer acid (HFPO-DA). It can also be used more informally to refer to the group of related fluorochemicals that are used to produce *GenX*. (3–5) As a replacement for perfluorooctanoic acid (PFOA), also designated at the time as C_8 - nomenclature and context to be detailed further below.

This class of man-made chemicals contains at least one perfluoroalkyl moiety in which all hydrogen (H) substituents on a carbon (C) atom have been replaced by fluorine (F). In addition to a perfluoroalkyl chain, designated the *tail*, most PFAS contain, at its extremity, a polar functional group, the *head*, which generally is a carboxylic acid, sulfonic acid, phosphonic acid, or phosphinic acid. (6–8)

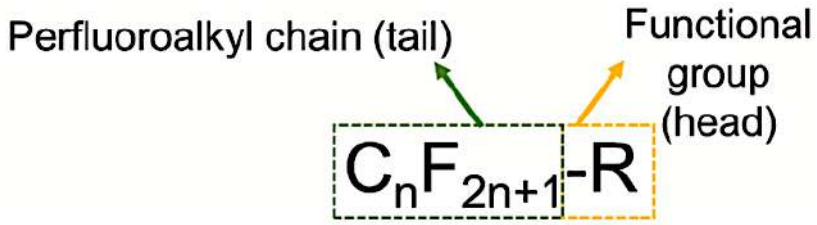


Figure 1. Fundamental structure of PFAS. (Adapted from Blake & Fenton, 2020)

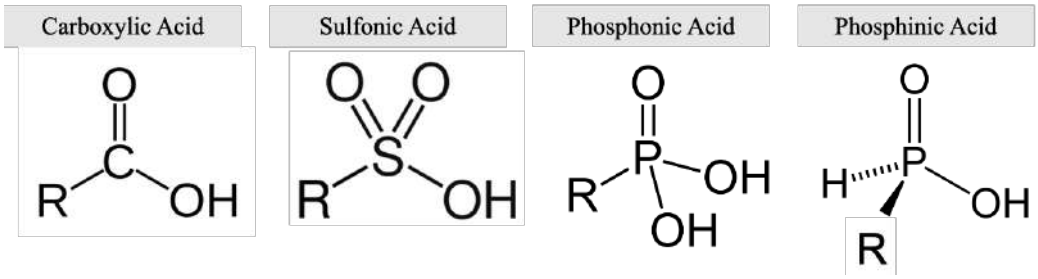


Figure 2. Most common head groups.

The high electronegativity and small size of fluorine make the C-F bond one of the strongest covalent bonds in nature. The large amount of energy required to break this bond is responsible for the stability of PFAS in the presence of oxidants and high temperatures as well as its resistance to chemical and biological degradation. Additionally, possession of a dual nature, hydrophobic and lipophobic, gives them unique, surface-active properties. (6)

Further substitution of a carbon atom only increases the bond strength due to the increasingly positive polarity taken by the carbon center, that results in the shortening of the bond length Increasing chain length is also associated with increasing lipophilicity and hydrophobicity. (9)

PFAS are individually characterized by carbon chain length, side group structure, and their history of use.

Table 1. Fluorine characteristics and subsequent proprieties on PFAS.

Fluorine Characteristic	Description	Result	Resulting Property of PFAS
High electronegativity	Tendency to attract shared electrons in a bond.	Strong convalent C-F bond.	**Thermal stability**
			Chemical stability Low reactivity
		Polar bond with partial negative charge towards F.	**Strong acidity** Low pKa (When paired with an acid functional group)
Low polarizability	Electron cloud density not easily impacted by the electric fields of other molecules.	Weak intermolecular interactions.	**Hydrophobic and lipophobic surfactant properties** (When paired with a functional group that is hydrophilic)
		Low surface energy.	
Small size	Atomic radius of covalently bonded fluorine is 0.72 A.	Shields carbon.	**Chemical stability** Low reactivity

Perfluoroalkyl acids (PFAAs) are sometimes described as *long-chain* and *short-chain* as a way to group perfluoroalkane sulfonic acids (PFSAs) and perfluorocarboxylic acids (PFCAs) that may present similarly in the environment. However, generalizations about PFAA behavior should not be made based only on chain length given other factors and proprieties with far more significance in that context.

Long-chain refers to PFCAs, with eight or more carbons (seven or more perfluorinated carbons), and PFSAs, with six or more (perfluorinated) carbons. *Short-chain* refers to PFCAs with seven or fewer carbons (six or fewer perfluorinated carbons) and PFSAs with five or fewer (perfluorinated) carbons. (10,11)

The so-called long-chain PFAS, which includes PFOA, perfluorononan-1-oic acid (PFNA), nonadecafluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoDA), perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), and perfluoroheptanane sulfonic acid (PFHpS), have been reported have more bioaccumulation potential and toxicities than short-chain PFAS. The tendency to substitute the former led to higher production, use, and consumption, and consequent exposure to the latter, which eventually translated into an increase in the serum levels of short-chain PFAS or other novel PFAS, although a decline had been noticed in serum levels of PFOA and PFOS, the most widely used long-chain PFAS, in some countries, since the early 2000s. (12)

Legacy PFAS include compounds with a longstanding history of use and/or long biologic/ environmental persistence, for which there are accumulating health data but that may be phased out or in decreasing use, whereas replacement PFAS compounds, often with minimal health effects data, are generally referred to as *Novel*, *Alternative* or *Emerging PFAS*. (8,13)

Since their genesis, PFAS have been incorporated into numerous industrial and consumer products due to their versatile spam of proprieties. Now in the 21st century, the vast majority of consumer products contain, and drinking water sources are now contaminated, with a mixture of PFAS. (13)

Under the European Chemicals Regulation, PFAS are classified as very persistent substances (vP), which clearly reflects the recalcitrant nature of these compounds. The vast majority of PFAS are therefore either non-degradable or ultimately transform into stable terminal transformation products, which are still PFAS. Based on concerns regarding the high persistence of these compounds and the scarce and limited knowledge of their properties, uses, and toxicological properties, it has been argued that the production and use of PFAS should be strictly limited. (2)

1.1 PFAS Families

PFAS encompass a vast universe of substances with very different physical and chemical properties, including gases (e.g. perfluorobutane (PFB)), liquids (e.g., fluorotelomer alcohols (PFTOHs)), surfactants (e.g., PFOS), and solid material high molecular weight polymers (e.g., PTFE). (14)

Its organization by families, as depicted in Figure 3, is therefore very useful, to group and associate compounds with similar physicochemical profiles and behaviors depicts that PFAS families may be divided into two primary categories: polymer and non-polymer.

1.1.1 Polymers

This class contains commonly used substances such as PTFE (*Teflon*) and ethylene tetrafluoroethylene (ETFE, trade name *Tefzel*).

However, most of the research on PFAS focuses on non-polymers, as they are more widely detected in the environment and thus are more likely to be the subject of government guidelines and regulations.

1.1.2 Non-Polymers

The non-polymer class is further divided into two subclasses: perfluoroalkyl and polyfluoroalkyl substances.

1.1.2.1 Perfluoroalkyl Substances

Perfluoroalkyl substances are fully fluorinated (perfluoro-) alkane (carbon-chain) molecules. Their basic chemical structure is a chain (or tail) of two or more carbon atoms with a charged functional head group attached at one end.

Commonly the functional groups are carboxylates or sulfonates, but other forms are also detected in the environment. (14)

1.1.2.2 Polyfluoroalkyl Substances

Polyfluoroalkyl substances are only partially fluorinated, containing a non-fluorine atom (typically hydrogen or oxygen) bonded to at least one, but not all, carbon atoms, while at least two or more of the remaining carbon atoms in the carbon chain tail are fully fluorinated.

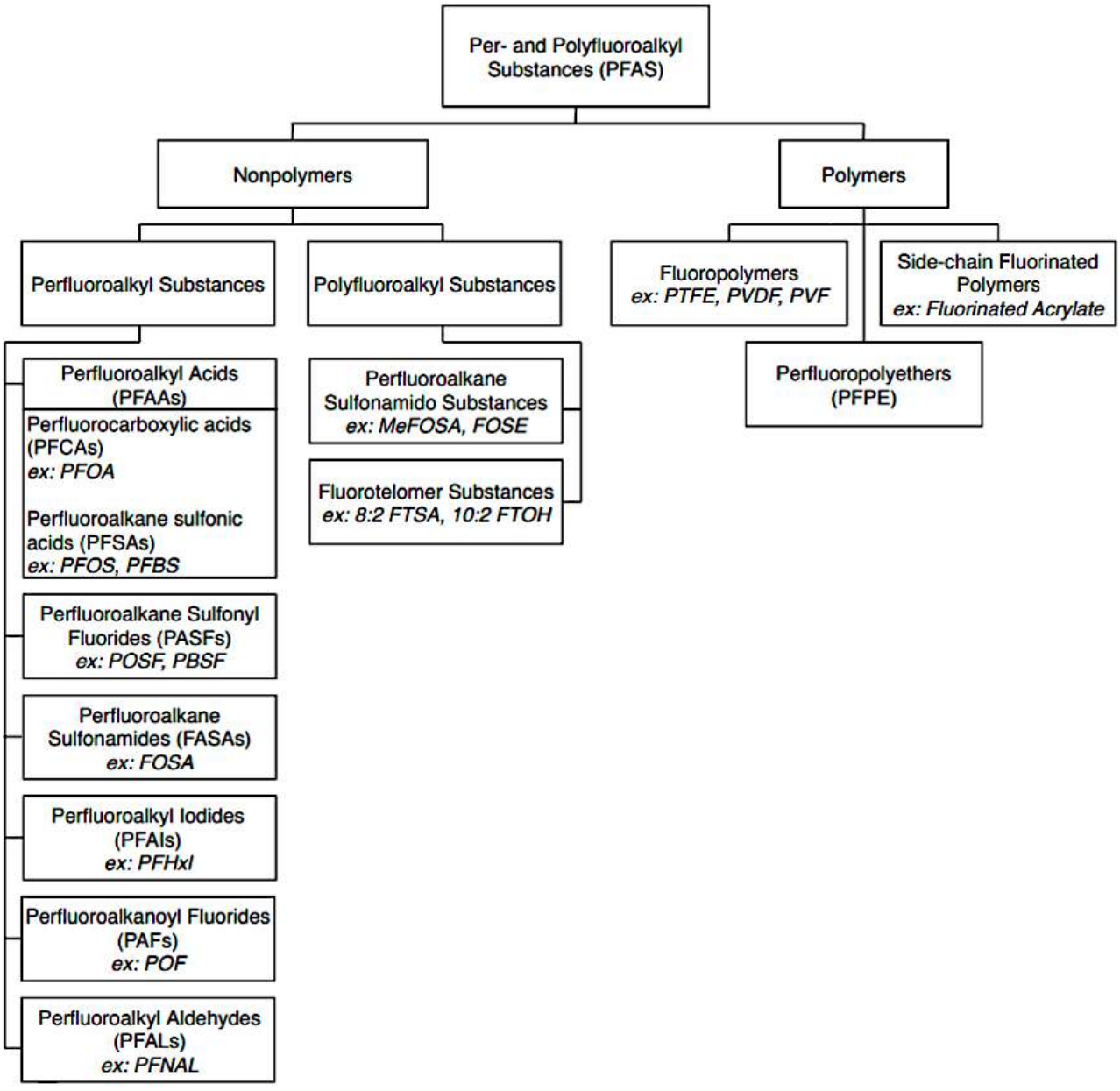


Figure 3. Classification tree of PFAS compounds into families. (From Meegoda et al., 2020)

1.2 Synthesis Methods

The creation of these non-polar, perfluoroalkyl chains is accomplished by two primary manufacturing processes: electrochemical fluorination (ECF) and telomerization.

1.2.1.1 Electrochemical Fluorination (ECF)

ECF is a process by which hydrogen atoms of raw materials are electrolytically replaced by fluorine atoms using anhydrous hydrofluoric acid, resulting in a finished product with both branched and linear isomers. Therefore, a foundational organofluorine chemistry method for the preparation of fluorocarbon-based organofluorine compounds, and seemingly the ideal method for the introduction of fluorine atoms into organic molecules since it can be carried out under mild conditions (85°C to 100°C) in a one-step procedure. (15,16)

Perfluoroalkyl radicals can be generated by the anodic oxidation of PFCAs, the reduction of perfluoroalkyl halides, and the electrochemical reduction of perfluoroalkyl iodonium salts or trifluoromethyl sulfonium salts. Those synthesized electrochemically can interact with small molecules such as CO_2, SO_2, and CS_2, and generate carboxylic, sulfinic, or dithiocarboxylic acids. These compounds will also have the capacity to react with electron-rich systems as well as charged nucleophiles, including aliphatic and aromatic thiols, oxygen-containing nucleophiles and carbon nucleophiles.

For many years the ECF process was the process of choice for the industrial production of long-chain perfluorinated substances, like PFOS and PFOA. However, for more than a decade, these substances are increasingly under criticism, since they are highly persistent, bioaccumulative, and have been consistently detected in the environment, being now globally recognized as contaminants of high concern. Due to these reasons, representative long-chain compounds, and their derivatives, are now strongly restricted. (16)

1.2.1.2 Telomerization

Telomerization reactions, pioneered by DuPont in 1942, usually yield low-molecular-weight polymers, designated *telomers*, or even monoadducts with well-defined end groups. Such products are usually produced from a *telogen* or transfer agent (X-Y), and one or more (n) molecules of a polymerizable derivative M (called *taxogen* or monomer) having ethylenic

unsaturation, under radical initiation conditions. (17) Telomerization is a 100% atom-efficient reaction whose final products are X–(M)n–Y telomers.

PFAS produced by telomerization has two important features, being that the process produces PFAS monomers that are -C_2F_4- units, or 100 Da apart, reflective of the *taxogen* building blocks. Secondly, the production of PFAS by telomerization produces linear isomers with little to no branched isomers. (15)

An additional step often occurs whereby the PFAS is further reacted with ethylene to produce a PFAS that contains a perfluorinatedalkyl chain, a -CH_2CH_2- bridge, and a functional group. (15)

1.3 Toxicological Hazards

Since the early 2000s, bioaccumulation of PFAS has raised concerns about their potential effects on humans and wildlife. (18) The description, for the first time, by Giesy and Kannan (2001), of the global extent of PFAS accumulation in marine organisms, terrestrial mammals, and seabirds, (19) exposed the gravity and extent of the contamination and toxic exposure inherent to the continued production and use of these compounds.

By this time, PFAS had become broadly distributed in the environment, with virtually all people living in industrialized countries already exposed to PFAS, with significant blood concentration levels, within the ng/mL range, of PFOA and PFOS. (12,20)

This discovery motivated and mobilized considerable scientific research efforts, focusing mainly on two chemical classes of PFAS - PFSAs and PFCAs - as well as their anthropogenic precursors. However, among the plethora of compounds that currently exist, belonging to the classes mentioned or to any others, only PFOA, PFOS, PFHxS, and PFNA, being the most used forms and constituting stable end products of other PFAS precursors, have been studied extensively. (18,21) Therefore, the toxicity of PFAS mixtures, as they appear in the environment, is still very poorly understood. (21)

It is known that various PFAS may affect similar organs and systems, but these effects occur at differing doses, depending on experimental design and the relative individual potency of each PFAS. A potential synergistic relationship between multiple PFAS has not been evaluated thoroughly, but in order to address this concern, several regulatory agencies have exercised a risk management strategy, instead of risk assessment, by assuming an additive

effect, as shown in some research (21), and applying an overall combined standard for the sum total of multiple PFAS. While perceived as protective, this risk management strategy lacks a scientific basis. (7,18)

1.3.1 Ecotoxicity and Environmental Concerns

PFAS human exposure scenario begins to portray itself through environmental exposure routes that include direct contact, with soil, sediment, and surface water; besides ingestion, diet seems to be the major human exposure pathway for some PFAS. Drinking water on the other hand has been verified to be the major exposure source in contaminated communities. (1,22) Inhalation is also a viable exposure route. (1,23) The prevalence, widespread distribution, and bioaccumulative potential and toxicity contribute to an alarming exposure spectrum, across all trophic levels, on a global scale. (1,6,9,14,22–24)

PFAS and their precursor compounds are released into the environment in a broad range of ways; including, during its manufacture and storage, in industrial point sources, plus the resulting emissions; landfilling of coated products such as food packaging and utensils, carpeting, or electronics; disposal of sewage sludge by land-spreading, resulting in contaminated biosolids, surface water and groundwater; use of fire-fighting foams, down to spillages and runoff incidents. (1,22) Once released, PFAS are recalcitrant compounds, meaning that, besides their heterogeneity as a group, due to their complex physical-chemical profile; their dual nature in terms of affinity for water along with surfactant properties play key roles in PFAS unique fate and transport characteristics. Besides persistent, due to generally not being broken down by either photolysis or microbial organisms in the soil or water; PFAS are very mobile in the environment, covering long distances from the source of their release, polluting soils and different water sources. (1,23) PFAS widespread surface and groundwater contamination became a matter of great concern, that soon took global proportions, with the finding of PFOS in wildlife by Buck *et al.* (2011) and Fu *et al.* (2014). (23,25,26) PFOS remains the most significant “PFAS-pollutant”, in fact, PFAS products in general remain in the environment for so long that scientists are still unable to estimate environmental half-life times.

PFAS have been identified in a wide variety of matrices, both aqueous (rain, snow, rivers, lakes, groundwater, tap water) and solid (sediments, sludge, soil). (7,22) *Long-chain* PFAS are more likely to attach to soil particles, (1,7,14,27,28) seemingly showing a preferential tendency to bind to carbon, which means that longer-chain compounds can be transported by

particulate matter, but can also accumulate in soil. *Short-chain* PFAS possess high aqueous solubility and relatively low adsorption potential, resulting in higher mobility rates in the environment, and are typically less bioaccumulative than *Long-chain* counterparts. (27,29) However*, Short-chain* final degradation products were deemed as transversally more mobile and more persistent in aquatic ecosystems. (30) For plants, *Short-chain* PFAS are typically more bioaccumulative; yet, for mammals and fish, the opposite is true, with *Long-chain* PFAS being more bioaccumulative than short-chain PFAS. (22) Moreover, volatile precursors are likely to be transported to remote regions via the atmosphere and then degraded to short-chain PFAS. (1,27)

Most ecotoxicological studies, as discussed further on, stick to a small subset of PFAS, primarily *Legacy* compounds, on a limited range of organisms, with most studies confined to single chemical exposures and developed to assess traditional toxicological endpoints (survival, growth, and reproduction). For aquatic wildlife, PFAS toxicity generally occurs in the order of parts per billion (ppb) for chronic, sublethal effects to tens of parts per million (ppm) for acute, lethal effects, with considerable overlap in effects concentrations in study duration, organism, and effect. Fewer studies are available for terrestrial invertebrates, but findings suggest toxicity occurs at similar concentrations. (22) Briefly, the threshold for the occurrence of adverse effects associated with PFAS exposure is much lower than the values obtained by research of this setting.

PFAS bind to serum albumin and fatty acid-binding proteins in the human body, unlike other previously enumerated lipophilic contaminants. (14,31) With albumin being a major protein in mammalian milk, breastfeeding emerged as a significant transport route between organisms. (32,33) rom plants, PFAS travel up the food chain through consumption, biomagnifying in top organisms, typically carnivorous predators. In a study conducted in the american state of Washington, Furl et al. (2010) demonstrated this trophic pathway assumption by identifying PFAS concentrations of up to 910 ng/g in osprey eggs, while concentrations in fish liver tissue did not exceed 530 ng/g. (34) In humans, ingestion is the main PFAS exposure pathway, occurring through exposure to consumer products, consumption of contaminated food, and contaminated water and breastmilk. The protein- binding capacity along with PFAS half-lives in humans, found to be longer than in any other mammal (Table 5), were catalytic factors of the recent regulatory reviews and updates in terms of restrictions limits to meat, fish, and eggs. (1,14) For wildlife exposures, a major challenge is adapting existing metrics for chemical accumulation to describe diverse PFAS. (22,35) As for the plethora of laboratory

rodent research available, it gives a wide database from which it's possible to derive PFAS toxicity reference values in mammals. Nonetheless, the studies are biased, mainly toward rodents, and the validity of applicability of these studies results to other taxonomic orders, namely human risk assessment, remains unclear. (36)

Past research on human exposure pathways has not included statistically representative population surveys, focusing on polluted locations, which frequently exhibit the greatest amounts of contaminants in the environment and biota. Presently, although the ingestion of PFAS is already considered the most relevant route of exposure, there's still limited evidence for other substances, including ones involved in food processing and packaging. Dermal PFAS exposure, from dust and personal care products (PCPs), also warrant additional consideration given the extremely high concentrations reported in these matrices. It's also worthy emphasizing the scarceness of data on indoor environment. (22)

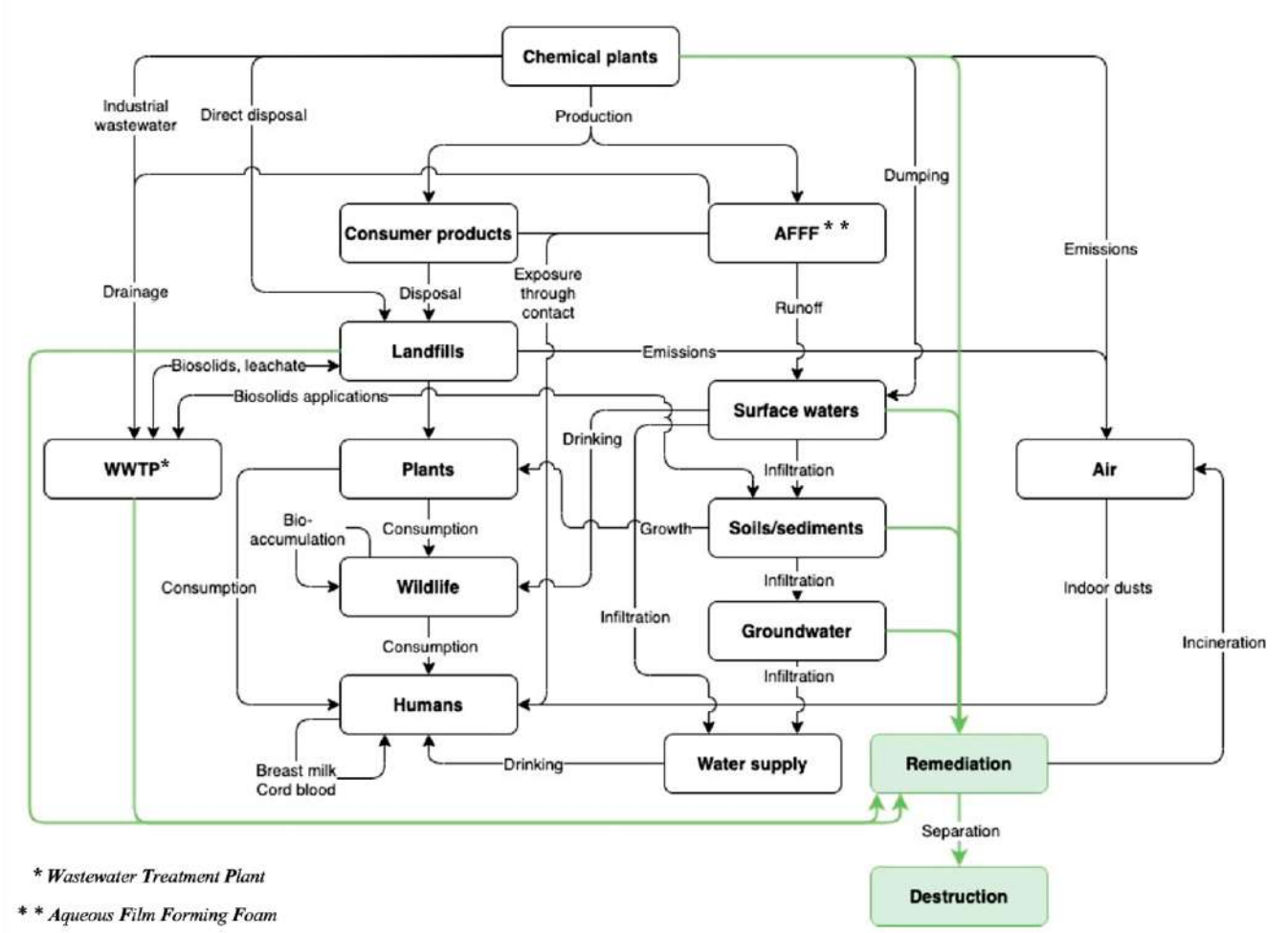


Figure 4. Schematic representation of PFAS release and exposure pathways. (From Meegoda et al. 2020)

Given the recalcitrant and hazardous nature of PFAS, employing practical and feasible techniques for their remediation is of urgent necessity, nerveless it is still a technically difficult, time-consuming, and costly process. (1,35–37) When traditional routes of wastewater treatment proved ineffective for complete eradication, various approaches have been proposed including the use of physical techniques - filtration, anion exchange resins, and adsorption - as this permit removal, but do not guarantee 100% destruction/eradication of PFAS from the separating wastes. Other advanced treatment techniques include hydrated electron-based reduction, sonochemical destruction, photocatalytic degradation, biological routes, plasma-based method, and electrochemical treatment. Nonetheless, certain impediments are still associated with these methods such for instance, high energy intake and complications and expenses in scale-up. (38)

Table 2. PFAS Treating Techniques (Adapted from Abunada el at. 2020)

Non-Destructive Treatment Techniques	Absorption
	Fractionation
	Ion Exchange
	High Pressure Membranes
	Reverse Osmosis
	Nanofiltration
Destructive Treatment Techniques	Advanced Oxidation Processes
	Electrochemical Oxidation
	Incineration
	Sono-chemical
	Biodegradation
	Photocatalytic Degradation
	Plasma-Base Treatments
	Hydrothermal Liquefaction

Table 3. Summary table of the cleaning and remediation processes for PFAS contamination.

	Process	*Basis Mechanism*	*Performance*	*References*
Non-Destructive Treatment Techniques	**Absorption**	Physical mass transfer process that uses Van der Waals and/or other weak ionic forces to bind the entire PFAS molecule to the surface areas of the adsorptive media.	Up to 95% removal efficiency.	(23,39,40,43)
	Fractionation	Processes that separate PFAS into different groups based on their chemical properties. Liquid chromatography, solid-phase extraction, and ultrafiltration.	Does not remove PFAS from the environment, but rather separates them into different groups for further analysis and characterization.	(41)
	Ion Exchange	Removal is an efficient technology for the remediation of PFAS-laden surface, ground and effluent wastewaters. This approach is more effective towards eliminating emerging short-chain PFAS which are not removed by carbon-based adsorption processes.	Up to 95% removal efficiency.	(40-43)
	High Pressure Membranes Membrane-based technologies that have been found to be extremely effective at removing PFAS from water		Research shows that these types of membranes are typically more than 90% effective at removing a wide range of PFAS, including short-chain PFAS.	(23,43,46,47)
	Reverse Osmosis	Reverse osmosis membranes are tighter than nanofiltration membranes, with both high-pressure membrane types, approximately 80% of the feed water passes through the membrane to the effluent (the treated water), and approximately 20% of the feed water is retained as a high-strength concentrated waste.		
	Nanofiltration			

	Process	Basis Mechanism	Performance	References	
Destructive Treatment Techniques	Advanced Oxidation Processes	Chemical treatment processes that use hydroxyl radicals to break down PFAS molecules. AOPs can be used to treat PFAS-contaminated water and soil. Various methods, including ultravioleta radiation with hydrogen peroxide, ozone and peroxide, or heat-activated persulfate can be used to generate the hydroxyl radicals.	Hydroxyl radical-based processes demonstrate up to 90% degradation of PFAS, but it needs to be pointed out that installation processes can be very costly.	(40)	Experimental Techniques
	Electrochemical Oxidation	Application of an electrical current through a conductive medium, triggering redox reactions with hydroxyl radicals as a sub-product. Electrochemical methods can be applied at ambient temperature and atmospheric pressure with no requirement of external reagents, still offering high energy efficiency, scalability, convenient set-up, and modularity, apart from easy and discontinuous operability at mild conditions. Additionally, when compared to other oxidative methods where supporting chemicals are essential for achieving superior efficiency, a small quantity of electrolyte is needed for the reaction to improve the process in terms of cost-effectiveness.		(23, 39,40, 43-45)	
	Incineration	Process that relies on high temperature chemical breakdown.	Is a disposal method rather than a full-scale treatment method.	(39,40,23)	
	Sono-chemical Treatment	Sono-chemical treatment is a physio-chemical treatment that uses ultrasonic waves (frequency ≥ 20 kHz) tipically to break down unreactive organic compounds. Used to treat water, sludge, soil, and sediment containing a large amount, and mixtures of various PFAS.	In laboratory setting showed excellent removal potential.	(23,40, 43, 48,52)	
	Biodegradation	Process that resorts to microorganisms to break down PFAS. It can can be used to treat contaminated soil.	N/A	(36,39)	
	Photocatalytic Degradation	Tecnique that uses light and a catalyst to break down PFAS. This processe generates hydroxyl radicals. Research has shown that it can be effective in removing PFAS from water and soil. The photocatalytic removal of PFAS from water under UV irradiation is most effective by using In2O3 as the catalyst, followed by Ga2O3 and TiO2.	The process has been shown to remove more than 98% of PFAS in 72 hours without oxygen. The photoreactor is also efficient for bacteria inactivation.	(39,49,43)	
	Plama-Based Treatment	Consists of the direct application of heat, by a Plasma-Reactor to the PFAS-contaminated solids (soil/sediment), breaking down the PFAS molecules. Plasma-based treatment of PFAS contaminated water successfully degrades PFOA and PFOS to below the EPA health advisory level of 70 ppt and accomplishes the near complete destruction of other PFAS within a short treatment time.	Despite na overall removal effectiveness,of up to 95%, plasma treatment depends on the individual properties of the influent being treated, and it's still an energy-cost-intensive process.	(38, 43, 50)	
	Hydrothermal Liquefaction	Promising technology for recovering energy from wastewater treatment sludge in the form of liquid biofuel. It is a thermochemical process that applies heat directly to the PFAS-contaminated solids (soil/sediment) to breaks down the molecules.	Hydrothermal liquefaction has been shown to be effective in degrading PFAS, but it requires extended reaction times, high temperatures and pressures and may produce hazardous byproducts.	(36, 43, 51)	

1.3.2 Human Exposure

Accumulating evidence has revealed that humans are universally exposed to PFAS. Pathways of human exposure include the intake of polluted food and drinking water, inhalation of (indoor and outdoor) air and dust, dermal contact and maternal to fetal transfer in utero, across the placental barrier, and through breastfeeding as neonates (8,39,40)

Due to its high solubility, most PFAS enters the human body through drinking, making it the largest and most significant source of PFAS exposure. (7,8,23) This in turn leads to formula-fed infants being thought to be the most highly exposed members of the human population due to their high water intake to body weight ratio. (8)

Their dual nature, hydrophobic and lipophobic, but especially their hydrophilic proprieties, allows PFAS to behave differently from many other organic pollutants when interacting with the human body. For instance, they have a strong tendency to interact with tissue and serum proteins such as albumin, which contributes to their distribution inside the organism and, opposing to many other bioaccumulative substances, PFAS are not stored in adipose tissue of humans and animals. PFAS primarily accumulate in the serum, lungs, kidney, liver, and brain. (1,8,41)

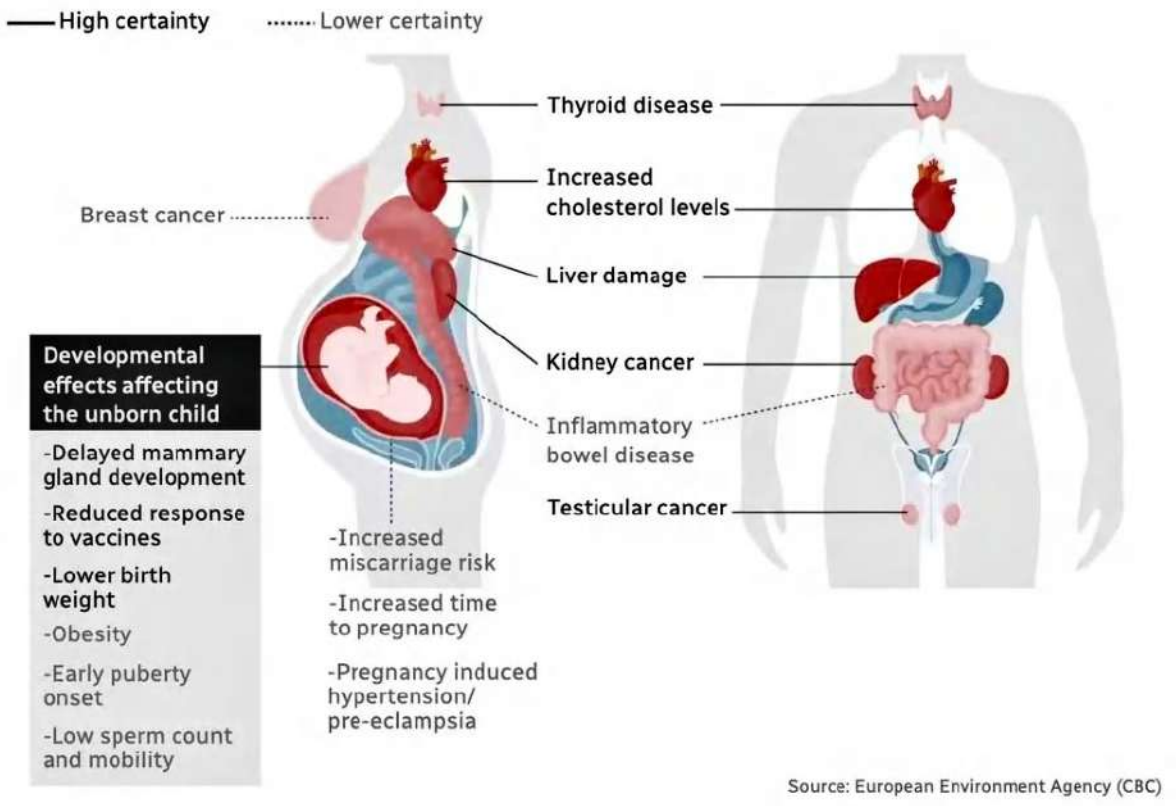


Figure 5. Outcomes of PFAS exposure on human health. (Adapted from Fenton et al., 2020)

PFAS are often referred to as forever chemicals due to the fact that they don't break down naturally and are resistant to environmental degradation and biological metabolization, remaining in the human body for a long period. PFOA, PFOS and PFHxS are the most predominant and frequently detected PFAS in human blood, with estimated serum half-lives of 3,8, 5,4, and 8,5 years (as seen Table 5). (12,39,41)

Epidemiological investigations have indicated a plausible association between PFAS exposure and various adverse health outcomes, with the most consistently observed and strongest evidence being of immune suppression (such as decreased vaccination response), liver function abnormality and hepatic toxicity (evidenced by higher levels cholesterol and liver enzymes), endocrine and metabolic disorders (increased glucose, dyslipidemia and thyroid disease), infertility, fetal growth restriction and resulting lower birth weight (which in turn resulted in later-on adverse impact infant/early childhood growth and development, physical and cognitive), cardiovascular diseases, bone mineral density reduction, *obesogenicity*, and tumor induction with an increased predisposition to the development of various types of cancers. (7,8,39)

A limited number of PFAS have been studied resorting to animal testing. Nevertheless, these studies show strong evidence that high exposures to PFAS can cause harmful health effects in animals, both early in life and as animals get older.

While there isn't a high acute exposure phenomenon to PFAS, and immediate health risks are low, long-term exposure leads to bioaccumulation of these chemicals, which is of particular concern in women of childbearing age since that increases the range of eventual exposure to the fetus and breastfed babies.

1.3.2.1 Hepatic and Metabolic Toxicity

The vast majority of PFAS are not known to metabolize or undergo chemical reactions within the body of living organisms. It has even been shown in some animal studies that PFAS can be excreted without forming any conjugates or metabolites. (42) Although metabolically inert compounds by themselves, they end up interfering with endogenous metabolic processes (41), which results in metabolic effects that compose one of the most significant outcomes of the exposure in question, given that it could induce a wide range of biochemical and physiological changes. Metabolic effects can thus be interpreted as possibly the underlying fundamental basis for other toxicological phenomena observed. Additionally, other systemic toxicities will most likely induce secondary metabolic effects, compromising the metabolic balance of the living organism. (13,41) Studies primarily focused on the hepatotoxicity of PFAS have shown that the interaction with cytochrome P450 (CYP450) may be a crucial molecular step to PFAS-induced hepatotoxicity.

Cytochrome P450 monooxygenases, or oxidoreductases, are a large family of cytoplasm-bound catalytic enzymes that are responsible for more than 75% of all biotransformation of pharmaceuticals. Humans encode 57 individual enzymes, grouped into 18 families. These enzymes are predominantly expressed in the liver, but they also occur in the small intestine, lungs, placenta, and kidneys. (43,44) Only six of these isozymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) metabolize 90% of drugs, with the two most significant being CYP3A4 and CYP2D6.

Besides its central role in the detoxification and metabolism of xenobiotics, it can also be pointed out that CYP450 enzymes are essential to the endogenous production of cholesterol, steroids, prostacyclins, and thromboxane A2. (44)

The hepatotoxic effects of legacy PFAS, like PFOS and PFOA, as well as some other long-chain PFAS, have been extensively studied. However, the mechanism of it has not yet been fully understood. The interaction, either induction or inhibition, of CYP450 enzymes has been proposed by Narimatsu et al. (2011), as potential key events leading to PFAS-related hepatotoxicity.

Amstutz et al. (2022) states that appears to exist a CYP-specific structure-dependent interaction of PFAS with the four investigated CYP isoforms, 2C19, 2D6, 3A4 and 2E1. Overall, it appears that PFAS toxicity increases with the chain length up to ten carbons. However, the mechanism of inhibition of CYPs by PFAS differed per CYP isoenzyme.

CYP2D6, CYP2C19 and CYP3A4 have shown similar inhibition patterns as their inhibition increased with increasing PFAS chain lengths, between six and nine carbons. These results imply that in addition to the restricted group of extensively studied PFAS so far, multiple novel PFAS may significantly alter the metabolic homeostasis of the exposed living organisms.

Table 4. CYP-PFAS interactions. (Adapted from Amstutz et al., 2022)

	CYP2C19	CYP2D6	CYP3A4	CYP2E1
PFOA	Non-Competitive Inhibition	Atypical Inhibition	Non-Competitive Inhibition	Inhibition
PFOS	Competitive Inhibition	Atypical Inhibition	Competitive Inhibition	Non-Competitive Inhibition
PFNA	Competitive Inhibition	Atypical Inhibition	Competitive Inhibition	N/A
PFDA	Inhibition	Atypical Inhibition	Competitive Inhibition	N/A
PFBS	Mixed Inhibition	Atypical Inhibition	Competitive Inhibition	N/A
PFHxS	Mixed Inhibition	Competitive Inhibition	Competitive Inhibition	Stimulation/ Increase in activity
PFHxA	Competitive Inhibition	N/A	N/A	Stimulation/ Increase in activity
PFHpA	Atypical Inhibition	Inhibition	N/A	N/A
3:1 FTOH	N/A	N/A	N/A	Inhibition
4:2 FTOH	N/A	Stimulation/ Increase in activity	N/A	N/A

Yet not only does the length of the carbon chain determine the alteration of the enzymatic activity, but the functional head group also plays a pivotal role in the PFAS-CYPs interaction. The head groups, in the case of a carbonyl group, appear to influence the inhibitory potential of PFAS when the carbon-chain length is below 8, whereas short-chained PFSAs

appear to be better inhibitors than their PFCAs counterparts. In contrast, differences in terms of affinity to the binding place site depend mainly on the PFAS chain length. For instance, below C8, the PFSAs can interact with a CYP2C19 regulatory site, while chain lengths of C8 or more will only interact with the active site, be it due to the size or lipophilicity is currently still unknown. Despite the global similarities in the inhibition patterns of the 2C19, 2D6 and 3A4 CYP-isoforms, there is a clear difference between the inhibition mechanism of CYP3A4 when compared to the previous ones. For CYP2D6 and CYP2C19, the type of inhibition appears to be concentration-dependent. However, the inhibition mechanism of CYP3A4 differs, with evidence of the not being concentration-dependent. (45) This can be explained by the morphology of the 3A4 binding site, both larger and more flexible (46) than the other two in question, which could result in a more pronounced inhibition phenomenon due to overall better access to the active site by the PFAS.

The CYP2E1 inhibition profile, interestingly, deviates from the rest. As seen in Table 4, 2E1 is the only CYP inhibited by PFTOHs, which was once again justified by the structure of its binding site. CYP2E1 possesses the smallest active site with a hydrophobic access channel. (45) As such, non-polar PFAS will have facilitated and, in turn, favorable access and interaction with the active site. CYP2E1 induction has since been linked, through animal studies, with increased oxidative stress, which itself causes hepatotoxicity, and sustained cell proliferation that eventually may lead to liver cancer or even Nonalcoholic Fatty Liver Disease (NAFLD). (45,47)

PFAS exposure, more concretely to PFOA, PFOS, and PFNA, has been consistently associated with the abnormal presence of liver enzymes, markers of liver injury, such as ALT, but also AST and GGT. (47,48)

The long fatty chain structures of some representative PFAS, along with the resultant Log P values, suggest that these compounds are hydrophobic, meaning they can easily penetrate through the cell membrane, but still, the exact mechanism behind PFAS hepatotoxicity remains unclear. One of the best-established hypotheses is centered around the activation of the Peroxisome Proliferator-Activated Receptor *Alpha* (PPARα), Annexe A1. , a major regulator of the hepatic lipid metabolism, and a main target for PFAS, resulting in liver inflammation and triglyceride accumulation. (47) Whereas PFOS, PFOA, and PFHxS have similar binding patterns to the peroxisome Proliferator-Activated Receptor *gamma* (PPARγ), with the whole structure of PFAS located in the pocket, close to the surface of the structure.

Alternate or even complementary mechanisms may involve the activation of Constitutive Androstane Receptor (CAR); down-regulation of Nuclear Factor Erythroid 2-related Factor 2 (Nrf2); and up-regulation of Nuclear Factor *kappa* B (NF-κB). An additional possibility, not fully explored yet, suggests that PFAS may cause steatosis by reducing the bioavailability of choline. (47) Epidemiological studies have also reported associations between PFAS exposure and high levels of cholesterol, bilirubin, and uric acid, further supporting a causal association.

1.3.2.2 Reproductive and Developmental Toxicity

As previously mentioned, despite the current phase-out of some legacy PFAS due to their proven environmental persistence and adverse health effects, alternative, short-chain and legacy PFAS mixtures continue to be produced and extensively used. Contaminated drinking water along with lifestyle factors such as diet and everyday products use contribute significantly to an individual's PFAS exposure. Exposure levels can also be determined, and intensified, by various demographic factors such as geography, race/ethnicity, age, sex, and occupation.

Trying to establish an association between sex/age and PFAS serum concentrations, Goralczyk et al. (2015) analyzed serum samples of men and women from Central Poland for 7 PFAS (PFHxS, PFOS, PFOA, PFNA, PFDA, PFUdA, perfluorododecanoic acid (PFDoA)), and found that the compound with the highest average serum concentration, in both sexes, was PFOS, measuring 18.49 ng/mL and 8.42 ng/mL, respectively. Later studies supported that generally, higher average PFAS serum concentrations were observed in men, except for PFHxS and PFDOA. (40,49)

The proper choice of animal model has gained relevance in subsequent studies, as the differences in half-life for elimination between the sexes within the same species were highlighted. C Lau (2012) estimated the half-life of PFOA elimination to be 3.8 years in humans, with little difference between genders, but in the rat, the half-life for elimination is 4–6 days in the male, but only 2–4 hours in the female. As for mice, appears to be the most adequate model since, like humans, there is little difference between sexes in the half-life of elimination (17 days in the female and 19 days in the male). (41)

Despite the increasingly concerning low rates of female fertility across the world, women's reproductive health consequences are still far less researched than those of men.

Table 5. Average serum/plasma elimination half-lives of some PFAS. (Adapted from: DeWitt, J. C. (Ed.), 2015)

Exposed Species	PFOA	PFOS	PFNA	PFDA	PFHxS	PFHxA
Rat	♂ 4-6 days / ♀ 2-4 hours	♂ 38-41 days / ♀ 62-71 days	♂ 30-31 days / ♀ 1-2 days	♂ 40 days / ♀ 59 days	♂ 29 days / ♀ ~ 1.8 hours	♂ 1-1.6 hours / ♀ 0.4-0.6 hours
Mouse	♂ 19 days / ♀ 17 days	♂ 36-43 days / ♀ 31-38 days	♂ 34-69 days / ♀ 26-68 days	N/A	♂ 28-30 days / ♀ 25-27 days	♂ ~ 1.6 hours / ♀ ~ 1.2 hours
Monkey	♂ 30-33 days / ♀ 21 days	♂ 132 days / ♀ 110 days	N/A	N/A	♂ 141 days / ♀ 87 days	♂ ~ 5.3 hours / ♀ ~ 2.4 hours
Rabbit	♂ 5.5 hours / ♀ 7 hours	N/A	N/A	N/A	N/A	N/A
Dog	♂ 20-30 days / ♀ 8-13 days	N/A	N/A	N/A	N/A	N/A
Cow	N/A	~ 114 days	N/A	N/A	N/A	N/A
Human	**~ 2.3 – 3.8 years**	**~ 5.4 years**	**N/A**	**♂ 12 years / ♀ 4.5 years**	**~ 8.5 years**	**~ 32 days**

Animal studies have demonstrated that PFAS target the female reproductive tissues directly, but also alter normal breast, thyroid, and hypothalamic–pituitary–gonadal axis (HPGA) function, (18,40) being the most common outcomes associated with PFAS exposure: increased time to pregnancy, hypertensive disorders of pregnancy (including pregnancy-induced hypertension and preeclampsia), gestational diabetes, excess gestational weight gain, low birth weight of the infant, delayed ossification and neurobehavioral effects. (7,8,35,39,50)

The placenta is a critical organ that ensures the maternal-fetal transfer of nutrients, oxygen, blood, waste, and xenobiotics. Is a foreseeable target of PFAS given its biological similarities to other known target tissues, such as the kidney and liver (1,41), but also due to its marked vulnerability to external agents (8), playing a crucial role in the etiology of pregnancy disorders related to PFAS exposure through an induced reduction in placental function - placental insufficiency, a condition in which the functional capacity of the placenta deteriorates and/or is limited, resulting in a reduced transplacental transfer to the fetus – or even an impairment on its development, significantly contributing to adverse pregnancy and birth outcomes. (51)

Except for representative compounds, there is an evident lack of information regarding the developmental toxicity of most PFAS compounds. Nevertheless, the information now available regarding perfluorobutanoic acid (PFBA), perfluorobutane sulfonic acid (PFBS),

PFHxS, and PFNA allowed to infer that pharmacokinetics and potency for activation of PPARα are determinant factors in developmental toxicity. Its molecular mechanisms are not yet fully understood but may include endocrine, lipid and sterol disruption, oxidative stress, inflammation, invasion, disrupted ratio of pro- and anti-apoptotic signaling, impaired trophoblast function, and epigenetic modifications. (8,52) Aside from being biologically quite complex, these mechanisms are compound-specific.

Activation of the PPARα and/or PPARγ appears to be the most plausible mechanism for the prenatal and postnatal growth effects. (53) PPARα is active probably through its effect on lipid oxidation, whereas PPARγ acts by increasing lipid flux into storage and by inducing secretion of beneficial hormones. (54) In mouse models prenatal PFAS-activated (PFOA) PPARα signaling, resulted in altered placental and fetal metabolic tissue development with subsequent impairments in fetal growth. (52) PPARs were also shown to be involved in modulating glucose and lipid metabolism or adipocyte differentiation (55).

Recent epidemiologic studies revealed inverse causal associations between maternal PFAS exposures and fatty acids during pregnancy (56) suggesting a negative impact of prenatal PFAS exposures on birth weight, through reduced availability of maternal fatty acids *in utero*. Whereas, PFAS-induced activation of PPARγ can lead to adipogenesis and inflammation (57), contributing to increased adiposity and risk of obesity in children and adolescents. (13)

Expression and activation of PPARα were shown to be necessary for mediating developmental outcomes, whereas early post-natal mortality caused by exposure was not dependent on the expression of PPARα. (12)

It has also been demonstrated that PFAS affect early-life reproductive endpoints, such as lactation and breastfeeding duration, pubertal endpoints, fertility and possibly fecundity. (40)

Grasty et al. (2003) animal trials showed that *in utero* exposure to PFOS contributed to postnatal lethality, with lung maturation, happening during the late stages of gestation, appearing to be a key event given its impact on functionality and subsequent survival. (58) Therefore establishing the concept of a prenatal window of susceptibility. (41) Further studies described increased thickness of the alveolar walls, being further speculated that the compounds themselves were interfering with surfactant function, more precisely with dipalmitoylphosphatidylcholine (DPPC), one of surfactant's major components. Representative

PFAS presented, at low concentrations, the ability to migrate from water into DPPC mono and bilayers, changing fluidity and phase transitions. (59)

Compounds with a short elimination half-life and low potency for PPARα activation showed lesser capacity for developmental effects. Furthermore, through i*n vitro* assays, was verified that PFAAs were not significantly capable of activating the human PPARα reporter. In general, among the PFAAs that do produce developmental toxicity in one or more laboratory species, prenatal PFAA exposure in teratology studies typically does not result in major malformations, with significant findings often associated with higher exposure levels that would result in maternal toxicity as well. (41)

While some health effects are possible to observe in certain animal models after PFAS exposure at human-relevant levels, this is not always the case. It's of great interest and importance in future *in vivo* research to focus on knowledge of potential PFAS exposure sources over time (housing history, behavioral habits, water sources etc.) menstrual history and cyclicity, pubertal and menopause timing, details of parity, and breastfeeding duration. (40)

Regarding specific sequels of prenatal and childhood PFAS-exposure in children, the following can be highlighted: impaired cognitive development, attention, impulse control, visual spatial abilities, and behavioral deviations. (60–62)

Worth noting that young children nowadays are likely to have higher PFAS burdens than adults due to cumulative exposure, not only due to the mothers exposure, via placental transfer and breastfeeding, which in itself is a substantial toxicological burden, but also through an higher water intake relative to body size (resulting in increased PFAS per volume), and more inhalation and/or ingestion of house dust due to their behavior. (12,13)

As for pubertal development, it's predictable PFAS to interfere with HPGA's normal functioning (63) but also to directly affect gonads, through their weak estrogenic or anti-androgenic actions, culminating, according to studies in female rats, in an advanced pubertal onset (64). Complementary references state that the effects of PFAS on childhood adiposity (65) can also trigger the metabolic and peripheral signals linked to an early pubertal onset. (66)

Notably, a variety of PFAS have been detected in human semen, reinforcing the magnitude of the overall exposure, but also posing a potential hazard to male fecundity. Epidemiological studies showed that representative PFAS exposure were adversely associated

with semen parameters in humans, including sperm count, morphology and motility. (39) Experimental results came to confirm exposure outcomes as testicular and epididymal damage and testosterone synthesis disorder, therefore impairing spermatogenesis and sperm quality. (67)

The underlying mechanisms of PFAS-related male reproductive toxicity remain largely unelucidated but current hypothesis reside in blood-testis barrier destruction, testicular apoptosis, altered testosterone synthesis, oxidative stress and Ca^{2+} influx, resulting in spermatozoa membrane composition and fluidity alterations. (39)

1.3.2.3 Endocrine Disruption

PFAS are known to act as Endocrine-Disrupting Chemicals (EDCs) – a class of chemicals that increases the risk of disease and/or negative health outcomes, across the entire lifespan, by altering the homeostasis and action of endogenous hormones or other signaling components of the endocrine system (65) - more precisely as a Thyroid Disrupting Chemicals (TDCs), impacting the hypothalamic-pituitary-thyroid axis and thyroid hormone production and metabolism (12,68), both directly and indirectly.

The academic community has settled on two major potential molecular mechanisms of PFAS-induced endocrine disruption: PFAS impact on adrenal and/or gonadal steroidogenesis and its interaction with nuclear hormone receptors (12,13), previously discussed and addressed from a different perspective. Nevertheless, according to Ghassabian *et al.* (2018), any step in the biosynthesis and secretion of thyroid hormones (THs) could potentially be affected by PFAS exposure. Several mechanisms could be envisaged, supported by *in vitro* and *in vivo* observations, including impairment of iodine uptake by thyroid cells through a competitive mechanism and/or direct inhibition of the sodium/ iodide symporter (NIS); interference with thyroglobulin synthesis; modification of thyroperoxidase (TPO) activity; interference with feedback mechanisms or with thyroid hormone biological effects through disruption of the signaling pathway, deiodinase enzymes activity or binding proteins. (60,69)

Overall, PFAS endocrine-disrupting abilities can lead to a variety of adverse health effects, with particular emphasis on altered hormone production, especially since THs are determinant for healthy growth and development. Abnormal adiposity being the most common sign of disturbed metabolic homeostasis.

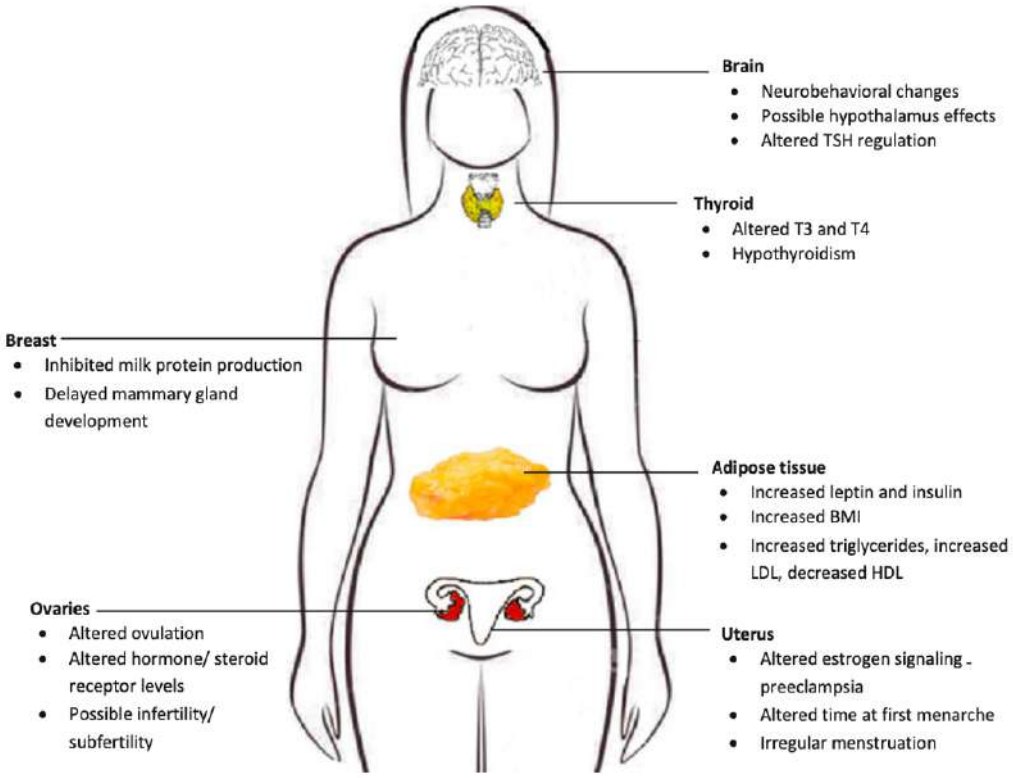


Figure 6. Endocrine targets of PFAS. (From DeWitt, J. C. (Ed.), 2015)

In-depth studies on the endocrine toxicity of representative PFAS showed that PFOS exposition results in suppressed estradiol, progesterone, and human chorionic gonadotropin secretion by placental syncytiotrophoblasts, the major transporting epithelium in the placenta, which will likely also disrupt normal placental development and function. As for PFOA, current research suggests that exposure may affect steroid hormone synthesis or act indirectly through ovarian effects. (21,51) These compounds also interfere with androgen production through down-regulation of CYP11A1, the enzyme that catalyzes the first reaction in the process of steroidogenesis in all mammalian tissues, converting of cholesterol to pregnenolone. (70) In addition, PFOA or PFOS proved to be cytotoxic to Leydig cells, responsible for androgen biosynthesis (71–73). Raising concerns regarding male exposure and its impact on important biological and reproductive processes such as spermatogenesis and sperm production, as well as sexual development and the maintenance of secondary sexual characteristics and behaviors, a consequence of altered testosterone levels. The same sort of health outcomes was verified for exposures to *alternative* PFAS. (43,74)

Although the possible biological mechanisms underlying PFAS-exposure-related abnormal growth, adiposity, and pubertal development during the fetal stage and the first years of life, are still not well understood, Lee et al. (2021) suggested several possible modes of action namely, PFAS-induced PPAR activation, alteration of sex and TH biosynthesis and metabolism, emphasizing PFAS ability to act as estrogenic or anti-androgenic substances. (12) Estrogen Receptor alpha (ERα) was also demonstrated to be a probable PFAS target. Unlike the binding patterns of PFAS in PPARα and PPARγ, the representative PFAS bind to the surface domain of ERα. The ligand-receptor binding is assured by hydrophobic bonds between representative PFAS and the nearby binding-site amino acids. (Figure 24. Ribbon (a) and surface (b) representation of PFAS in the structure of ERa (From: DeWitt, J. C. (Ed.) (2015) Toxicological effects of perfluoroalkyl and polyfluoroalkyl substances)) The docking representations in annex, also allow visualizing how PFAS bind to the active site of PPARα and PPARγ (A1. or bind to the region close to the active site of Erα (A2. , hampering it for endogenous ligands to bind to the catalytic sites, if the PFAS have occupied those regions. Thus, PFAS disturb the function of these receptors by interfering with the ligand-receptor interactions. (41)

Furthermore, experimental, and epidemiological studies have reported a link between PFAS-exposure and consequent “metabolic-endocrine-disorders” such as altered glucose homeostasis, body weight, and insulin resistance, normally accompanied by clinical pictures of increased fasting glucose, incident diabetes, or even metabolic syndrome. (21) All possibly due to PFAS physical-chemical proprieties allowing these compounds to mimic fatty acids, having deep effects on homeostasis. These may include diabetes-related cardiovascular mortality, claimed as a major complication associated with environmental exposure to PFAS; Major liver toxicity, identified in preclinical research aiming to clarify possible mechanisms by which PFOA can interfere with the regulation of hepatic glucose metabolism. Through exposition of HepG2 cells, an *in vitro* model human hepatocyte, researchers evaluated glycogen synthesis, glucose uptake, and Glut-4 glucose transporter translocation upon PFOA, and insulin exposure, coming to the conclusion that the acute exposure of human hepatocytes to PFOA was associated with the impairment of insulin receptor signaling, resulting in reduced glucose uptake and glycogen synthesis. (75) And even PFAS-related alarming neurodevelopmental toxicity or its accumulation in bone tissues, causing impaired bone development. (21)

Despite these findings, the gaps in current knowledge take even bigger dimension considering the complex biological interplay between PFAS endocrine target tissues and the resulting long-term health effects. The outcomes listed have also been shown to be deeply dependent on the compound(s) itself(s), level and type of exposure, age, underlying comorbidities, developmental period of exposure and geographic area.

1.3.2.4 Immunotoxicity

The immune system is well known to be particularly sensitive to the effects of environmental and day-to-day exposures to representative PFAS. (41) In general, elevated serum PFAS levels, in adults and children, correlate with observed PFAS-related immunotoxicity, including decreases in serum vaccine titers, and C-reactive protein (CRP) levels, alterations in immunoglobulins (Igs) levels, and even, other effects such as changes in specific cell populations in lymphoid organs, or alterations in lymphoid organ weights were observed; increases in antinuclear antibodies, and clinical states like asthma, common cold, and gastroenteritis. (41) However, once again, the different mechanisms by which PFAS generate immunotoxicity are not completely understood, and it's most likely a result of multiple interlinked pathways. (13,20)

By 2013, Grandjean and Budtz-Jørgensen estimated Benchmark Doses (BMDs) for the representative PFAS, already under the assumption that previously published BMDs were not adequately protective of children or of the public, given potential suppression of immune responses. A BMD of 1.3 ng/mL for PFOS and 0.3 ng/mL for PFOA were established. Additionally, they calculated a BMDL (Benchmark Dose Level, or lower one-sided 95 % confidence limit of the BMD) of 1 μg/L and a Reference Dose (RfD) of 1 ng/L. (76)

In vitro and *in vivo* studies, some of them to be further described below, led European Food Safety Authority's (EFSA) panel (2020) to, when reviewing the physio-pathological mechanisms of PFAS-related immunotoxicity, focus on, firstly a consensus, in an epidemiologic context, regarding cytokines core involvement, but also on the abnormal transactivation of several nuclear receptors: PPARs, Nuclear Factor *kappa* B (NF-κB), CAR, Nrf2, SXR (Steroid and Xenobiotic Receptor) and RXR (Retinoid X Receptor). Stronger evidence supported the first two. (20)

Although contradictory effects have been reported, most PFAS tested clearly induced NF-κB. This nuclear receptor is found in almost all animal cell types, and known to regulate

inflammation processes and cellular apoptosis, participating in adaptive and innate immune responses to *stimuli* such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized Low Density Lipoprotein (LDL), and bacterial or viral antigens. (20,77)

Whereas PFAS direct effect on the modulation of TNF-α (Tumor Necrosis Factor *alpha*), IL-6 (Interleukin-6) and IFN-γ (Interferon *gamma*), was consistently reported *in vitro*, sustaining its effect on PPARs gene expression. (20,78) Modulation of gene regulation appears to occur mainly *via* activation of PPARα, and to a lesser extent, of PPARγ, but still, species differences, including sex-related ones, affecting PPARs-expression, difficult it's interpretation as an underlying mechanism in humans. (41)

Due to its widespread expression on lymphocytes, hepatocytes, cardiac cells, and microglia, as well as its subsequent actions - regulation of gene expression via lipid pathways, increasing permeability of mitochondrial membranes, affecting glucose regulation, cell proliferation, and inflammation - (PFCs) activation of PPARs was established as the primary immunotoxic pathway. (41,79)

Animal studies have attempted to shed light on how exposure to some PFAS may be related to decreased splenic and thymic levels of circulating WBCs, but the current available WBC-population-specific studies are insufficient to provide a clear picture of the potential effects of PFAS-exposure on immune cell phenotypes at human-relevant levels. When extrapolating rodent studies to humans, it is blatant that suppression of adaptive immunity occurs at exposure levels that are within a reasonable range for humans. Human hepatic PPARα expression is only one-tenth that of rodents. (80) and in general, males endure longer half-life elimination rates when compared to females (41,81). The underlying mechanism contributing to the different toxicokinetic factors in females as compared to males is not completely understood but is believed to involve hormonal differences influencing the uptake of these chemicals. (7) Thus far, there have been very few studies on human cells.

Brieger et al. (2011), isolated peripheral blood mononuclear cells (PBMCs), from 11 volunteers, and tested for NK cytolytic activity following *in vitro* exposure to PFOA or PFOS. The viability of PMBCs was not affected, yet suppression of NK cytolytic activity and increasing TNF-α *post* lipopolysaccharide (LPS) stimulation was observed. (20,82)

Dong et. al (2013) highlighted the immunotoxicity of PFAS corroborated by a positive association, previously reported by both US National Toxicology Program (NTP) and EFSA, between serum eosinophil counts among asthmatic cases and PFAS concentrations in children.

Additional epidemiological studies were carried out aiming to describe associations between PFAS exposure and peripheral white blood cell (WBC) counts. (20,83)

Oulhote et al. (2017) prospectively examined 56 children to determine associations between persistent *post*-natal exposures to five PFAS (PFOS, PFOA, PFHxS, PFNA, PFDA) and the differential counts of WBCs Higher PFAS serum levels at the 18th month and the fifth year were linked to higher basophil counts in the, then, 5 year-old children. (20,84)

Lopez-Espinosa et. al (2021), proposed that PFAS exposure was positively associated with absolute lymphocyte counts and Natural Killer (NK) cells, through evidence from peripheral WBC counts in a human population in the Mid-Ohio Valley, USA, exposed to PFOA, *via* drinking water and background exposure to other PFAS. Still, no associations were reported related to significant changes in the populations of LynB and LynT ($LynT_H$ and $LynT_C$), neither relatively to CD4/CD8 ratios). Even so, exposition to PFHxS demonstrated the strongest association with lymphocyte counts. (20,85)

All evidence available leaded EFSA (2020) and EPA to agree upon the definition of adverse effects on the immune system, specially worrying in children, due to its stronger link with immune dysfunction, as the most critical in human health risk assessment, based on strong evidence, from animal studies, showing suppression of antibody responses from vaccinations and moderate evidence for suppression of antibody responses in humans. (20,41) Limitations of the mentioned studies include social and behavioral factors that may have influenced the correlation between PFCs and age-related immune responses. (41)

By 2021, the US Agency for Toxic Substances and Disease Registry reported, based on epidemiological evidence, that not only the representative compounds, PFOA and PFOS, but also PFHxS and PFDA, were associated with a decreased antibody response to vaccines. (20)

1.3.2.5 Neurotoxicity

Although the brain is not a dominant tissue for PFAS accumulation, adverse effects of PFAS exposure on brain functions have already been identified. Data available from several biomonitoring and exposure studies about PFAS uptake, accumulation, distribution and impacts on the brain, show that PFAS enter and accumulate in the brain with varying efficiencies, due to the massive heterogeneity characteristic of this group of compounds. When compared, long-chain PFAS are shown to cross cerebral barriers more effectively than short-chain PFAS and end up accumulating in the brain of humans and wildlife species.

The brain stem, hippocampus, hypothalamus, pons/medulla and thalamus are the dominant locations for PFAS accumulation. (86)

The accumulation and distribution of PFAS in the brain may lead to toxic effects in the Central Nervous System (CNS), including PFAS-induced neurological abnormalities and behavioral and cognitive disorders (35,62,84), with animal exposure experiments, showing both short-chain and long-chain PFAS could induce such outcomes. However, evidence is mixed when it comes to human data. (13,86)

Although long-chain PFAS have been phased out and been replaced by diverse emerging PFAS, they are still present in tissues and the environment. Information on the neurotoxicity of both long-chain and emerging PFAS is still lacking.

As reviewed above, disruption of thyroid hormone function during fetal life is very likely to affect the neurodevelopment of the fetus. (86) THs are crucial for normal brain development, being essential for orchestrating the processes of neurogenesis, migration, synaptogenesis, and myelination. Indeed, insufficient thyroid hormone levels at critical times of human neurodevelopment can induce long-term intellectual and behavioral impairments. Moreover, THs regulate metabolism, throughout the entirety of life, primarily through its actions in the brain, white fat, brown fat, skeletal muscle, liver, and pancreas. (60) With a positive association, with varying levels of evidence, between PFAS exposure and several neurological traits such as impulsivity, attention-deficit/hyperactivity disorder (ADHD), behavioral problems, or poorer executive function. (60,62,84)

Various *in vitro* PFAS exposure experiments have been conducted, mainly on hippocampal neurons, to further explore the mechanisms of PFAS brain toxicity. Hippocampal neurons are promising objects of investigation since the hippocampus is the main brain area related to learning and memory, besides being one of the dominant brain areas for PFAS accumulation. (86) The specific pathways underlying PFAS-related neurotoxicity remain to be fully uncovered, but the current two primary mechanisms for it are based on recent understanding of the PFAS effects on calcium homeostasis and consequently, on neurotransmitters.

Calcium (Ca^{2+}) is responsible for mediating multiple neuronal processes, such as synaptogenesis, proliferation, apoptosis, and neurotransmitter secretion. Calcium signaling plays a major role in regulating cell functions, including innate and adaptive immune responses.

(20,86) Macrophages, neutrophils, NK cells, dendritic cells, and mast cells, are dependent on tightly controlled Ca^{2+} signaling for their activation and effector functions like degranulation, cytokine release, phagocytosis, cytotoxicity, ROS production and inflammasome activation. Therefore, an altered Ca^{2+} regulation in immune-relevant cells may result in various autoimmune, inflammatory and immunodeficiency syndromes.

Various PFAS exposure studies have reported effects on calcium homeostasis in neurons, more specifically a PFAS-induced calcium levels increase in neurons, due to either extracellular calcium influx or calcium store release from the mitochondria and the endoplasmic reticulum. Studies have also reported PFAS-induced alteration of calcium-dependent down-stream signaling molecules - Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII), cAMP-Response Element Binding Protein (CREB) and calcineurin (CaM).

PFOS was shown to increase the expression of CaMKIl and phosphorylated CREB in adult male rat cortex and hippocampus. Likewise, PFNA induced increases in intracellular calcium concentrations and CaMKII expression in rat pheochromocytoma-12 (PC12) cells. Expression of CaM in rat hippocampal neurons significantly increased in situations, of separated exposition, to both compounds. *Long-chain* PFAS neonatal exposure triggered an increase in the level of growth-associated protein-43 (GAP-43), synaptophysin and tau protein in mouse hippocampus and cerebral cortex. (86) All the above could translate into oxidative stress that could ultimately lead to cell apoptosis.

The second most studied potential mechanism of PFAS-related neurotoxicity is neurotransmitter dysfunction. It can only be deduced as to the extent of the impact caused by such exposure, given that the neurotransmitters showing greatest impact *post*-exposure are dopamine, glutamate and acetylcholine.

Research on PFAS absorption, accumulation, distribution, and toxicity in the brain is gaining attention, but, as with the many other areas of investigation associated with PFAS-exposure, many critical gaps remain. PFAS may enter the brain through induced BBB disassembly and/or relying on transporters, but once again, even the entry mechanism seems to be compound-specific. Although some of the main storage sites have already been identified, its entire route there, as well as secondary accumulation sites, remain to be thoroughly elucidated. Indeed, this kind of experiment is invasive to brain tissues and structures. As a result, to reduce the risk associated, and to make PFAS-related brain studies more accessible, a

focus for the future is selecting adequate alternatives (such as cerebrospinal fluid and hair) that can accurately reproduce PFAS brain concentrations. (86)

1.3.2.6 Cardiotoxicity

Cardiovascular disease (CVD) is one of the most common and worrying medical conditions nowadays, being the lead cause of death globally (WHO, 2021). Occurrence and severity appear to be deeply associated with exposure to environmental pollutants, such as PFAS. Although some PFAS, including PFOS, PFOA, and PFHxS, have been phased out of manufacture and use, due to cardiometabolic health effects (87), the effects of PFAS-bioaccumulation in the body, environment, and food chain, remain not fully understood, let alone prevented. Several novel PFAS alternatives have emerged, raising similar concerns, seeming to play prominent toxic roles in CV outcomes that might be milder, similar, or even more severe than legacy PFAS. (88)

Extensive epidemiological evidence suggests that accumulated serum levels of legacy PFAS may contribute to an increased risk of CVD and its subclinical signs, such as cardiac toxicity, vascular disorder, hypertension, and dyslipidemia, with strong evidence for a link to obesity. (88,89) Focusing on epidemiological and experimental studies exploring the relationship between PFAS exposure and thromboembolic cardiovascular disease, recent evidence indicates, how new-generation PFAS, alike legacy compounds, could represent a possible risk factor for thromboembolic events, with the implication of platelet-centered mechanisms, in populations chronically exposed to PFAS. (21,90) Similarly, among data gathered by Wittkop *et al.* (2021), increased concentrations of a new, short-chain PFAS, perfluoroheptanoic acid (PFHpA), stood out due to its apparent association with impaired vascular function among outpatients without CVD. (87)

The underlying biological mechanisms may include oxidative stress, signaling pathway disturbance, lipid metabolism disturbance, and so on. Studies even describe a strong concern regarding how dual PPARγ/α activators, which is the case with PFAS, as previously discussed, may be cardiotoxic, posing other risks including carcinogenesis and kidney damage (91)

Future efforts is transversely recommended to conduct more in-depth CV toxicity assessments of PFAS, to explore more effective surveillance, prevention, and treatment strategies, accordingly. (88)

1.3.2.7 Carcinogenic and Tumorigenic Activity

According to the rest of the data available on PFAS exposures has, through the most heterogeneous pathways, been linked to cancer, and as discussed above, besides a variety of other central causes of mortality in humans and many wildlife species.

Studies of occupational exposure to relatively high levels of PFOA and PFOS have not shown consistent evidence for an association with any specific cancer type. With studies among workers, from 3M (92) and DuPont (93), reporting, nor similar nor strong associations with significant health outcomes. Obviously, no sustained plausible association was disclosed for exposures at low levels either.

The toxicologic evidence for the carcinogenicity of PFAS has mostly come from studies on exposure to a few PFAAs - PFOA, PFOS, and undecafluorohexanoic acid (PFHxA). Each produced a different response. PFOA induced the tumor triad associated with peroxisome-proliferating chemicals, including liver, pancreas adenomas, and testicles. Rats exposed to PFOS developed mainly liver tumors. (94). While there are no associations between PFAS and cancer that have been both evident and consistent across studies, there is some evidence for an association of PFOA with testicular cancer, a very rare, non-lethal type of cancer. Two studies of this association, one cohort (Barry *et al.*, 2013) and one case-control (Vieira *et al.*, 2013), coincided in finding a strong positive exposure-response for this cancer, besides kidney, prostate, and ovarian cancers and non-Hodgkin lymphoma, which was also for some of the enumerated outcomes, supported by animal data. (93,95)

Evidence for kidney cancer is also suggestive, especially in PFAS-exposed areas. Shearer et al. (2020) found, through a case-control study, a strong exposure-response trend with PFOA, not appreciable for other PFAS. (96) These results come to contradict those reported by Raleigh *et al.* (2014) since kidney cancer was taught not to be associated with PFOA exposure in the high-PFOA-exposure occupational cohort of 3M workers. (92) With the exception of the liver cancer excess recently reported in Italian employees exposed to PFOA, there is no evidence for such links to be drawn about PFOA-induced liver or pancreatic cancer, despite the fact that these tumors have been connected with PFOA exposure in rodent studies. (97).

Based on consolidated epidemiological evidence *legacy* compounds, namely PFOA and PFOS, considering to-date quantified exposure, now low and fortunately decreasing, the prospect of reaching a point of carcinogenic repercussions seems contained. (41) Therefore, mechanistic studies, gain relevance, for a better understanding of *short-chain* PFAS

toxicological potential and possible role in cancer etiology, as these are the ones that still represent an unparalleled challenge at regulatory level.

Pieozan *et. al* (2022) treated normal human breast epithelial cells (MCF-10A) with 500 pM to 500 μM of PFHxS, PFHxA, *GenX*, perfluoro 3,6 dioxaoctanoic acid (PFO2OA), heptafluorobutyric acid (HFBA) and PFBS for 72 hours to investigate potential effects on cell proliferation and neoplastic transformation. Only the exposure of 100 μM PFHxS prompted significant alterations in regulatory cell-cycle proteins (cyclin D1, CDK6, p27, p53 and ERK) and cell proliferation, presumably through activation of CAR and PPARα. This compound triggered cell malignance through induction of epithelial cell proliferation, and promotion of migration and invasion potential, by altering the activity of cell-cycle regulators, adhesion proteins (E-cadherin and β-integrin) and histones. That said, there's clear scientific proof that while exposure to *alternative* PFAS has generally been shown to be less hazardous than to *legacy* ones, compounds like PFHxS, a ubiquitous environmental contaminant, with toxicological profiles of similar or even more severity, stand out. (98) Overall, the evidence from epidemiologic studies of PFAS in relation to cancer is strongest for testicular and kidney cancer but remains limited.

To date the only large, long-term cohort of an exposed community with data on incident disease is the PFOA-exposed cohort in the mid-Ohio valley (93,95), but similar studies could be possible in other locations. Two such large general population cohorts, with good exposure contrasts, have been formed in Ronneby, Sweden (primarily PFHxS and PFOS; by Li et al., (2018) (99)) and Veneto, Italy (PFOA; by Pitter et al., (2020) (100)), whose results and have certainly contributed greatly to the knowledge we have acquired since then.

It's important for individuals to be aware of potential sources of PFAS exposure, adding another possible "defensive" everyday action, and implement filtration systems for drinking water, besides being cautious with product use and consumption.

1.4 Current Regulatory Approach

Concordance between animal and epidemiology studies is limited. Pharmacokinetic differences impact human equivalent dose calculations and contribute to uncertainties regarding the interpretation of associations between human health effects and relatively low, exposure or serum, concentrations of a specific PFAS. Nevertheless, the persistence, mobility, and toxicity of several PFAS justifies their classification as Substances of Very High Concern (SVHC), and their identification and management as a chemical class under the European Union's REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) legislation. Even though all "non-essential" uses of PFAS are gradually being phased out, continued use and release will culminate in increasing exposure concentrations, and consequently increasing probabilities of occurrence of, known and unknown, effects. Once adverse effects are identified, the exposure-related toxicity is not easily reversible, which can be expected to be technically challenging, energetically demanding, and costly for society, as is evident in the efforts made to date to remove PFAS from contaminated land and drinking water sources. (18)

The Stockholm Convention on Persistent Organic Pollutants was adopted in 2001 and entered into force in 2004. At its fourth meeting in 2009, the Conference of the Parties amended Annexes A, B, and C to the Convention to include alpha hexachlorocyclohexane; beta hexachlorocyclohexane; chlordecone; hexabromobiphenyl; hexabromodiphenyl ether and heptabromodiphenyl ether; lindane; pentachlorobenzene; perfl uorooctane sulfonic acid, its salts and perfluorooctane sulfonylfluoride; and tetrabromodiphenyl ether and pentabromodiphenyl ether. (101–103) Meanwhile, the United States Environmental Protection Agency (USEPA) PFOA Stewardship Program (2006) set a goal of a 95% reduction in emissions of PFOA and related chemicals by 2010 and eliminating them from emissions and products by 2015. Needless to say, a significant portion of these goals remained unfulfilled. Even though the reduction in the use of numerous PFAS has been achieved, these programs lacked capacity to manage the numerous others with similar characteristics that are still being produced and used, suggesting that substitution does not eliminate the concerns with PFAS, but rather amplifies it. (35)

According to the European Chemicals Agency (ECHA), REACH Regulation included PFOS manufacturing, placing on the market, and use in the EU for more than 10 years already, under EU's Persistent Organic Pollutants (POPs) Regulation. But only in June 2019, the first substance was added to the SVHCs Candidate List - 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionic acid, its salts, and its acyl halides (HFPO-DA), a short-chain

PFAS substitute for PFOA in fluoropolymer production. Followed, in January 2020, by the proposal of restriction of perfluorobutane sulfonic acid (PFBS), a replacement of PFOS, and its salts. In June 2022, Norway proposed a restriction on PFHxS, its salts, and related substances, and in July, PFOA was finally banned under the POPs Regulation. In December 2021, Germany proposed a further restriction for PFHxA.

More recently, in Helsinki, on 13th January 2023, the national authorities of Denmark, Germany, Netherlands, Norway and Sweden prepared and submitted a proposal to ECHA, EU's largest-ever chemicals prohibition, in support of statements made in the EU Environment Council, back in December 2019, stating that, despite efforts made thus far, the PFAS-associated risks are not adequately controlled and must be addressed throughout the EU and the European Economic Area (EEA). Two options to restrict usage were suggested: full ban, including importation, "with no exemptions, and an 18-month transition period after the regulation enters into force"; or "with use-specific, time-limited exemptions that would carry an 18-month transition period and a five- or 12-year derogation period, depending on the application". Presently, EFSA is the stage of evaluation and a six-month consultation period that started in mid-March 2023. In that same month, PFHpA and its salts; and one month later, C_9–C_{14} PFCAs, their salts, and related compounds, have been restricted under REACH Regulation. (35,102,104) In short, the newly proposed European Union PFAS ban covers chemicals, mixtures, and articles with 25 ppb or more of a specified PFAS or 250 ppb of a combination of PFAS. (14)

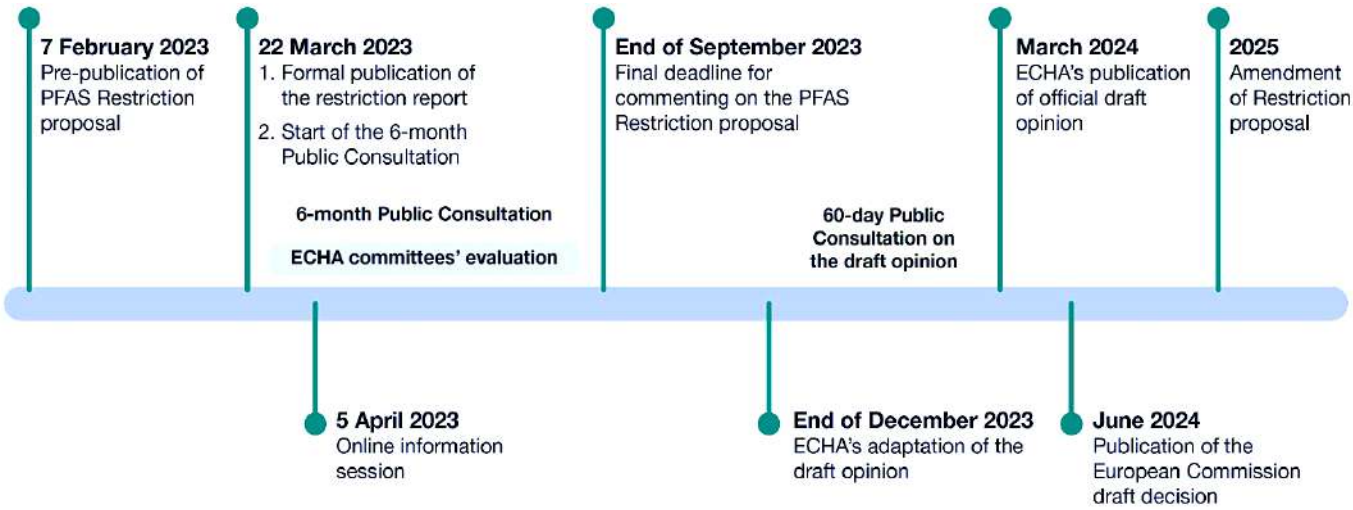


Figure 7. PFAS legislative panorama in the EU/EEA. (From (Cockcroft et al., "EU reach restrictions: How to prepare for the proposed PFAS restriction", 2023 [Online])

Of utmost importance in regulatory matters are also the two pieces of legislation that, at the beginning of 2023 came into full force in the EU, limiting human exposure to harmful amounts of PFAS. In September 2020, with the Commission Regulation (EU) 2022/2388, EFSA established a group Tolerable Weekly Intake (TWI), based on data such as described above, for the PFAS with the identified most concerning bioaccumulation rates - PFOA, PFOS, PFNA, and PFHxS - of 4.4 ng/kg/week. (35,105) Highlighting that this limit was set taking into account that the presence of PFAS in food, as highly persistent substances, is normally not directly linked to food production processes, even though it occurs through the use and disposal of PFAS-containing products, but instead its release into the environment. The main contributors to human dietary exposure are certain vegetables and drinking water, the significant contributions of fish and seafood, meat and meat products, eggs, milk and dairy products, with reported concentrations in the subnanogram to low nanogram per gram range. (22,105) On the other hand, Directive (EU) 2020/2184, The Recast of the Drinking Water Directive, EU's main law on drinking water, which took effect on 12 January 2021, places a limit, of all PFAS, of 0.5 μg/l in drinking water. (35,106)

The US approach to PFAS is also worth mentioning, as it is to some extent pioneering in terms of regulatory matters of PCPs. On December 29, 2022, US President Joseph Biden signed into law the Modernization of Cosmetics Regulation Act (MoCRA) of 2022 as part of the Consolidated Appropriations Act, 2023. This legislation marks a notable change in the Food and Drug Administration's (FDA) regulation of PCPs. MoCRA substantially expands the authority of the Food and Drug Administration (FDA) to promulgate new regulations over cosmetics and initiate enforcement against manufacturers and distributors of cosmetic products that present health risks. (107) Although PFAS cannot be regarded as the only consideration underlying the enactment, it certainly factored into the development of this law, which represents a significant uptick in the manner of FDA regulation of the cosmetics industry.

The collection of data now accessible, including those discussed in more detail above (*1.3 Toxicological Hazards)* served as a basis for the enunciated legislation. The literature generally has reported ecological thresholds much higher than those for human health. However, synthesis of analytical standards for newer PFAS has lagged far behind their production and release, and thus nontargeted methods, suspect screening, and total fluorine assessments have all emerged as essential tools for understanding the total burden of PFAS in the environment, humans, and wildlife, more exhaustive data is needed, particularly for food items, PCPs, dust, drinking water, and wildlife tissues that contain PFAS. (22)

A more defensive and preventive regulatory approach, knowing PFAS act as environmental trigger, seems the correct approach to the process of decision making on risk management. Relying on an overall *weight of evidence* (WOE) approach, from multiple studies, that may preferably include tissue sampling, toxicity testing, and field surveys. Limitations may be placed on the value of evidence of the latter's interpretations, due to the presence of conmmon co-contaminants (such as polychlorinated biphenyls (PCBs), pesticides, or metals), physical factors, or ecological stressors (predators, food availability) that may confound results or influence conclusions. (35) One could even argue that the current drinking water limits are indeed based on developmental and subchronic toxicity, with this reference levels most likely not encompassing immunotoxicity derived using serum levels. If a BMD based on serum vaccine titers (i.e., RfD 1 ng/L) were acknowledged, then current limits may be several hundred-fold too high.

The bottom line being that ECHA acknowledges that a holistic group approach to regulatory assessment and risk management needs to be explored, committing to phase out all PFAS, allowing, until late 2030s, use only where they are proven to be irreplaceable and essential to society.

2 Materials and Methods

2.1 Laboratory Research Objective

This experimental approach aims to corroborate existing evidence while further contextualizing these findings, especially when viewed in terms of possible “core events” in the pathways of immunosusceptibility and impairment. Subject matter and design follow current research trends by attempting to bridge the knowledge gap regarding to, particularly, the immunocompromise and immunotoxicity caused by PFAS exposure, through alteration of monocytes overall state and function.

Monocytes are a type of white blood cell that plays a critical role in the immune system's defense against infections and other diseases. They are produced in the bone marrow through hematopoiesis, involving the differentiation of hematopoietic stem cells (HSCs) into various blood cell types. Monocytes are derived from a common myeloid progenitor cell, which is a precursor to several myeloid cell types, including monocytes, granulocytes (neutrophils, eosinophils, and basophils), and megakaryocytes (which produce platelets). Through several stages of differentiation – myeloblast, monoblast, promonocyte – a common myeloid progenitor matures into monocytes. Monocytes are released from the bone marrow into the bloodstream, where they circulate for several hours to days before migrating to tissues throughout the body. This cell uses the bloodstream for patrolling reasons, migrating into tissues in response to infection, inflammation, or tissue damage. Monocytes can differentiate into macrophages or dendritic cells in tissues, which are crucial for phagocytosis, antigen presentation, and immune regulation.

Specifically regarding the THP-1 lineage, a spontaneously immortalized monocyte-like cell line, derived from the peripheral blood of a childhood case of acute monocytic leukemia (M5 subtype) (108), was selected for the present study of monocytic viability upon exposure to PFAS. THP-1 cells have been widely used in recent assessments of PFAS safety profile, through immunotoxicity investigation of PFAS-exposure effects on cytokine release in THP-1 cells, being found that the exposure effect occurred pre-transcriptionally. (109) Another study revealed that PFAS can activate the innate immune system via the AIM2 inflammasome, and that the exposure impact was reduced in THP-1-derived macrophages lacking AIM2 inflammasome components. (110) Exposing THP-1 cells in a laboratory setting might then be

useful for a variety of purposes, with the main goal for this study being the assessment of the toxic effects of PFAS exposure on a monocyte-lineage cell. THP-1 cells were exposed to 50µL of 100 µM PFAS – four different, individually treated, PFCAs, PFOA, PFNA, PFDA, and PFUdA – with cell viability being determined through an MTS Assay. The aim was to verify the intrinsic toxicity of PFAS to such basic degree as to analyze the effect of basis PFAS compound properties, such as carbon chain length and degree of fluorination, with data expected be strengthened with the exclusion of the contribution of the influence of the head group to the overall compound toxicity and outcomes, due to all the tested compound presenting a carboxylic acid group in that position.

The vast majority of available evidence relates to exposures of a magnitude that in reality are only observed in very specific situations. The present study, however, focuses on exploring the effects of a single 72-hour exposure to four individual PFAS, PFOA, PFNA, and PFUdA, at a concentration which, although still significantly higher than the realistic average, is much lower than that studied in most available research.

THP-1 cells were exposed to the test PFCAs for 72 hours, and afterwards incubated for 4 hours with MTS reagent at 37°C 5%CO_2. Viable cells will generate a signal that is proportional to the number of viable cells present. When cells die, they rapidly lose the ability to convert the reagent in a detectable product. (111) This study, like many others of the kind, is based on that same fundamental distinction.

2.1.1 THP-1 Cell Line

THP-1 is a human monocytic cell line, isolated from the peripheral blood of a 1-year-old male patient suffering from acute monocytic leukemia (108,112). It was established by Tsuchiya *et al.* in 1980 and has been extensively used to this date to study monocyte/macrophage functions and mechanisms, signaling pathways, and nutrient and drug transport, having become a common model to estimate the modulation of monocyte and macrophage activities (113), as it was the first cell line to be known that demonstrated characteristics of activated macrophages without exogenous stimulation. (112) Early studies indicated that THP-1 cells resemble primary monocytes and macrophages in morphological and functional properties, including differentiation markers. (113)

Monocytes and macrophages belong to the innate immune system, whose primary functions include the recognition of foreign pathogens, such as bacteria, fungi and viruses, through contact of their surface structures with various types of Pattern Recognition Receptors (PRRs), proliferation to increase the number of cells able to eliminate pathogens and stop infectious processes, production of pro-inflammatory chemokines and cytokines, recruiting effector cells to the site of infection, and anti-inflammatory cytokines, when the infection is under control and, phagocytosis, to engulf and digest pathogens. (113)

When activated, becomes adherent, being designated as A-THP-1, exhibiting, besides remarkable phenotypic changes, an increased phagocytotic and bactericidal activity, HLA-DR expression and antigen presentation capacity, secretion of monokines, as well as a tendency to form multinucleated giant cells. In addition, the derivate cell line has enhanced constitutive production of IL-1β and increased complement receptor and FcγR expression. (114)

A-THP-1 cell line demonstrates, and maintains, characteristics of an activated state without exogenous stimulation. (112,114).

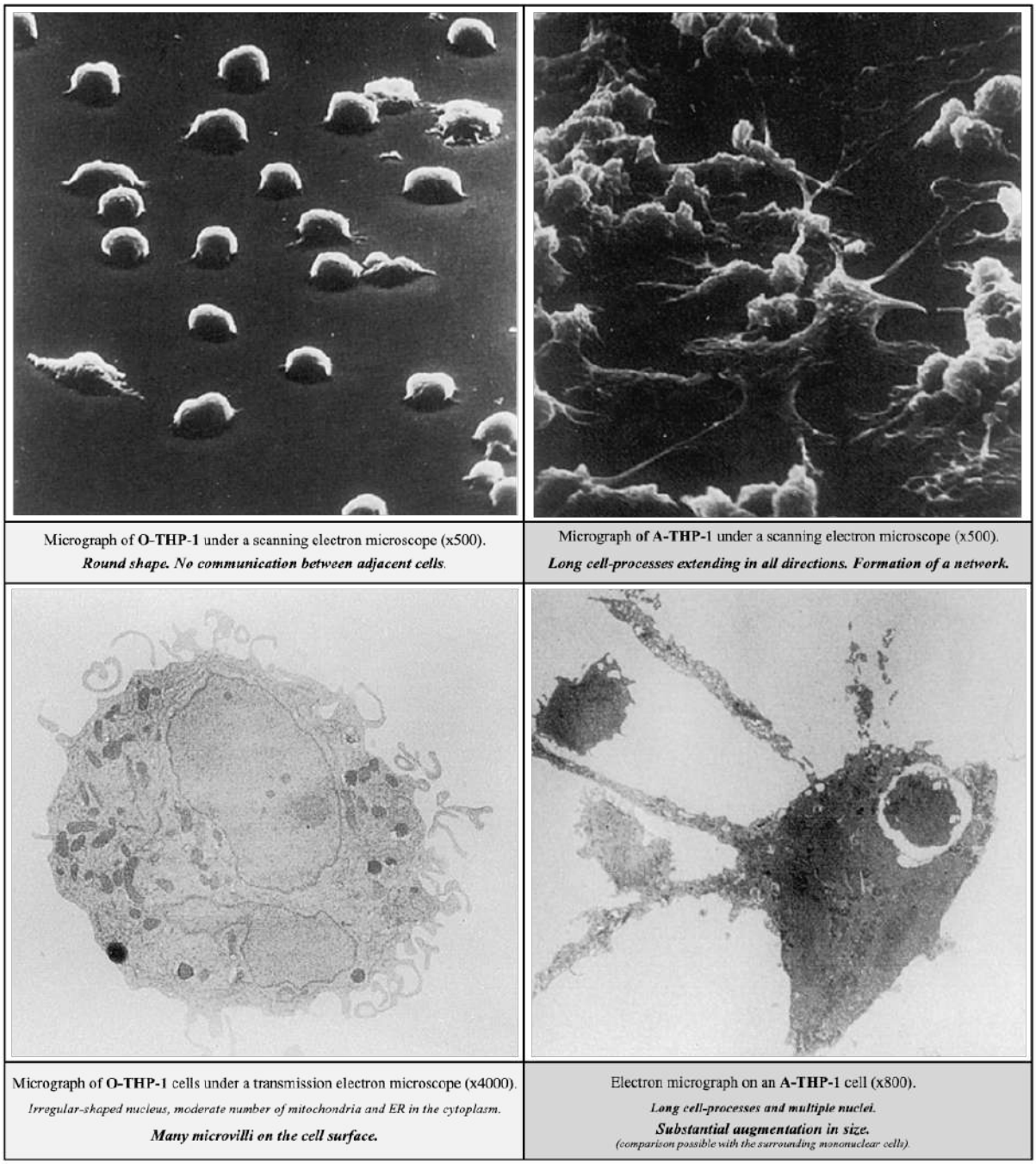


Figure 8. Structural observational comparison between O-THP-1 and A-THP-1 cell lines. (Adapted from Tominaga et al. 1998)

Macrophages are plastic and heterogeneous cells, as a consequence of differentiation mechanisms that vary in responsiveness to *stimuli* and due to tissue localization. Distinct $LynT_{CD4+}$ subsets, among other cell types, play major roles in regulating the differentiation of THP-1 cells into macrophages, and posterior polarization into distinct activated phenotypes. (113) IFN-induced or classically activated M1 macrophages, with a pro-inflammatory phenotype; whereas IL-4, IL-13, and IL-10 induce alternatively activated M2 macrophages,

with an anti-inflammatory phenotype. M1 macrophages are cytotoxic to pathogens and tumor cells, prompting etoposide-induced cancer cell apoptosis; while M2 macrophages have a clear protective effect on cancer cells. (115,116) Nevertheless, studies showing that M1 and M2 THP-1 macrophages have the same expression profiles as polarized primary macrophages. (116)

This characterization is based on observations that M1-like macrophages play a role in producing pro-inflammatory cytokines and mediators, providing host defense against microorganisms and in tumor regression by stimulating a LynT$_H$ *Type 1* (Th1) -driven immune response. Expression of TNF-α, IL-1β, IL-6, IL-8 and IL-12 genes, and expression of PRRs, such as *Toll-Like Receptors* (TLRs) and *NOD-Like Receptors* (NLRs), have been reported to be up-regulated during M1 activation. Alternatively, M2 type macrophages participate in the resolution of parasite infection, tissue modeling, immune-regulation, allergy and tumor progression by stimulating a Th2-driven immune response. M2 macrophages have been further classified into three subsets: M2a induced by IL-4 or IL-13, M2b induced by exposure to immune complexes/TLR-agonists or IL-1-receptor, and M2c induced by IL-10. (117) These characteristics of M2 subtypes are hypothesized to be related to specific functions such as killing of pathogens, immune regulation and tissue remodeling. (113)

The advantages of this cell lineage, over monocytes or macrophages derived from PBMCs include its average doubling time (PDT) of around 35 to 50 hours, reflecting a much higher growth rate than PBMC-derived monocytes, whose PDT stands between 5 to 7 days. Under growing conditions with the use of RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), THP-1 cells can quadruple within three and a half days. THP-1 cells are free of infectious viruses or toxic products, which qualifies the cell line for a biosafety classification level of BSL 1, making it reasonably simple and safe to work with. The fact that THP-1 is an immortalized cell line that can be cultured *in vitro* for up to 25 passages, i.e., for approximately 3 months, without changes in cell sensitivity or activity. Regarding its storage, THP-1 cells can be stored for several years, and by following an appropriate protocol, this cell line can be recovered without any significant effects on its monocyte–macrophage features and cell viability. In opposition, PBMC-derived monocytes require inflammatory mediators, such as IL-1β, TNF-α or LPS, to prevent apoptosis, and the availability of this kind of cells is often limited since they cannot be stocked in liquid nitrogen. And, the homogeneous genetic background of THP-1 minimizes the degree of variability in the cell phenotype, facilitating the reproducibility

of findings. However, this homogeneous background can also be rendered as a disadvantage, as observed effects may end up being identified as genotype-dependent.

Unintended effects from in vitro differentiation of THP-1 macrophages, for instance, up-regulation of specific genes might exaggerate mild effects of specific *stimuli*, however, this might be less of an issue if strong *stimuli*, e.g. drugs or chemical compounds, are present. Another drawback is that the malignant background and the cultivation of cells under controlled conditions might result in different sensitivities and responses compared to normal somatic cells in their natural-physiological environment. Nevertheless, there's an overall advantage in using the THP-1 cell line, since the high variation from individual donors became a common problem associated with the application of PBMC-derived monocytes. (113)

Leukocyte isolation from buffy coats must be performed within a week after the blood is withdrawn. Monocytes from buffy coat of healthy adults can vary in concentration from 2% to 8% of PBMC, which amounts to about 200 to 800 monocytes per microliter (µl) of blood. An absolute monocyte count above 10%, or 800 per mm^3, is considered high. Contamination with other blood components (such as platelets) should be taken into account.

Table 6. Typical white blood cells (WBCs) counts in the average healthy person. (From Administração Central do Sistema de Saúde, I.P. (ACSS) Laboratory Reference Values Sheet)

	Differential WBC Counts					
Total WBC	*Segmented* Neutrophils	*Band* Neutrophils	Eosinophils	Basinophils	Lymphocytes	Monocytes
(4.5 – 11) x10³/µl	54 - 62%	3 - 5%	1 - 3%	0 – 0.75%	25 – 33%	3 – 7%

2.1.2 Handling Procedure

ATCC (American Type Culture Collection)'s THP-1 cell line was used for the present study's purpose. TIB-202™ was shipped frozen on dry ice in an appropriate cryopreservation vial. Upon receipt, after checking all containers for any leakage or breakage, the frozen cells were removed from the dry ice packaging and immediately placed at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use, concordantly with the supplier's instruction. The base medium for this cell line is RPMI-1640 Medium, which was supplemented with 10% FBS, L-glutamine, and 1% antibiotic/anti-mycotic, to obtain the complete growth

medium (GM). Avoiding excessive alkalinity of the medium, particularly, during the "recovery period" of the cells, it's of major importance. Prior to the addition of the vial contents, the culture vessel(s) containing the GM, at appropriate temperature, must be placed in the incubator for at least 15 minutes to allow the medium to reach its normal pH (7- 7,6).

For assurance of the highest level of viability, the culture was thawed as soon as possible upon receipt, by gentle agitation of the vial in a 37°C water bath for just around 2 minutes. When fully thawed, it was removed from the water bath and externally decontaminated with 70% ethanol. From this point on, all procedures involving the handling of the cell culture were carried out under strict aseptic conditions.

To initiate the frozen culture, the vial was transferred to a centrifuge tube containing 9 mL of GM, spin at approximately 125 x *g* for 5-7 minutes. Once the culture has been dispensed into the required number of culture vessels, incubate it under 37°C 5% CO_2 conditions.

2.1.3 Suspension Subculturing Procedure

For roughly 10 minutes, the Vertical Laminar Flow Unit (VLFU) was set to Stand-By mode with the UV light on. Meanwhile, the GM flask was removed from the refrigerator and placed on a heated bead bath to balance out temperatures with the cell culture.

Before carrying out the subculture procedure, in this study case, given the growth properties of the THP-1 lineage, the cell culture, in its culture vessel, was examined through microscopic observation. Cell culture observation and analysis, throughout the whole study, were done on ZEISS's smart microscope for cell culture and research, Axiovert 5, equipped with Axiocam 208, which automatically adjusts sharpness, brightness, and white balance, offering high-quality live images.

At the lowest magnification - 10× ocular magnification x 4× objective magnification, summing to a total magnification power of 40× - the GM was checked for evidence of microbial contamination. Bacterial contamination would appear as small, shimmering black dots within the spaces between the cells; yeast contamination, as rounded or budding particles; while fungi would present thin filamentous mycelia. The morphology was also observed, as an indicator of viability and global state. Cells in suspension culture grow either as single cells or in clusters. Viable cells appear round and refractile, whereas dead cells appear smaller and darker. Occasionally, a portion of the cells may attach and grow on the side of the culture vessel and appear round or flattened. The percentage of attached cells varies with the culture conditions

and the cell density. Cellular debris may also be observed in healthy cell populations. Cell propagation in suspension has several advantages over propagation in monolayer. Subculturing is a simple matter of dilution, with little or no growth lag after splitting a suspension culture, as there is with a monolayer culture, because in the latter there's a factor of physical *stress* associated with the process itself. (118). For THP-1 cell line the recommended subculture seeding concentration ranges from 2,0-4,0x10^5 cells/mL.

Once the THP-1 cell culture was found fit for subculturing, it was momentarily introduced back into the incubator, as the VLFU was switched to *Normal* mode, enabling appropriate lighting and electricity inside the chamber. The protective glass was raised just enough for the materials, previously sterilized externally with 70% ethanol, to be inserted inside – Eppendorf *Safe-Lock* Tubes (0,5 mL, 1,5 mL and 2,0 mL), adequate micropipettes, and disposable points, sterile disposable pipettes, Trypan Blue (TB), and a Neubauer Chamber with a lamella, and finally, the GM flask, now at approximately room temperature, and the cell culture vial, brought out of the incubator, containing the thawed cell culture, $P_{THP\text{-}1}0$. An automatic pipette, tube supports, the vortex and disposable bins are permanently kept inside the VLFU.

About 1mL of cell culture was transferred to a 1.5 mL Eppendorf tube, using a sterile 2mL pipette, promoting resuspension of the culture through the pipetting itself (avoiding the formation of bubbles), followed by vortex of the Eppendorf for a couple of seconds. 20 µL were transferred to another 1.5 mL microtube Eppendorf tube, to which the same volume of TP was added, using a 100 µL micropipette. This tube was vortexed, and with a 10 µL micropipette, 10µL of vitally colored cell culture were drawn into the previously prepared counting chamber, by capillary action. The hemocytometer was then placed under the microscope at a total 100× magnification. The number of cells in each quadrant was accounted for with the help of a hand tally counter clicker. The average number of cells *per* quadrant was multiplied by the dilution factor, 10^4 cells/mL, since the cells have not yet been diluted. The total volume of the subculture was established to be 5mL. so, using the dilutions formula, it was possible to calculate the volume needed to be extracted from the aliquot of $P0_{THP\text{-}1}$ – $C_1 \times V_1 = C_2 \times V_2$ – where C_1 will correspond to the calculated cell density of the culture to be diluted; V_1, the volume to be drawn from it; C_2 the appropriated *new* initial concentration, optimally between 2-4 x10^5 viable cells/mL, and the already mentioned V_2 (5 mL).

Afterwards, the volume of GM (V_{GM}) required must be determined by subtracting V_2 from V_1. V_{GM} was transferred to a new culture vessel, properly sterilized, and brought inside the

VLFU, to which was then added the V_1, to obtain the desired C_2. The culture vessel should then be completely closed, solely to ensure that no leakage happens, while the subculture undergoes observational examination under the microscope, just to be slightly open before being put on the incubator (37ºC, 5% CO_2), to guarantee the appropriate ventilation of the THP-1 cell culture.

Note that THP-1 subculture should be done when cell concentration reaches $8x10^5$ cells/mL, never allowing the culture to exceed a density of $1x10^6$ cells/mL. The suspension cell subculturing should be done, every 2 to 3 days, respecting recommended GM renewal spam.

2.1.4 Viability Cell Assay: MTS Tetrazolium Assay

Evaluation and interpretation of cell viability are crucial, regardless of the kind of cell-based experiment intended to be developed. Cell viability is expressed as the percentage of living, healthy cells in a sample, and it may be used to assess the physical and physiological health outcomes in specific cells in response to external *stimuli*, chemical agents, or drugs. (119)

Some of the fundamental methods to assess cellular viability include tetrazolium reduction, resazurin reduction, protease markers, and ATP detection. The first three measure some aspect of general metabolism, or an enzymatic activity, as a marker of viable cells, requiring incubation of a reagent with a population of viable cells. Under most standard culture conditions, viable cells incubated for a certain period with a specific reagent generate a signal that is proportional to the number of viable cells present. When cells die, they rapidly lose the ability to convert the reagent in a detectable product. This study, like many others of the kind, is based on that same fundamental distinction. As for the ATP assay, the addition of assay reagent immediately ruptures the cells, thus not requiring an incubation period. (111)

There's a variety of tetrazolium reagents, the most common being MTT, MTS, XTT, and WST-1. MTT is positively charged and readily penetrates viable eukaryotic cells whereas MTS, XTT, and WST-1, more recently developed, are negatively charged and, even though such charge has been found to contribute to an increase of solubility in cell culture medium, does not allow such facilitated intake. The latter class is typically used with an intermediate electron acceptor that can penetrate viable cells, that becomes reduced in the cytoplasm or at the cell surface, facilitating the reduction of the tetrazolium. The CellTiter 96® AQueous One Solution Reagent, used in the present study, contains MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and PES (phenazine ethosulfate). PES has been found to increase chemical stability, enabling the combined preparation of

tetrazolium compounds and an electron coupling reagent. (111,119) As for MTS, also known as *Owen's Reagent*, it's a tetrazolium compound bioreduced by cells into a colored formazan product, soluble in most culture mediums. This conversion is presumably accomplished by NADPH or NADH, produced by dehydrogenase enzymes in metabolically active cells. (111)

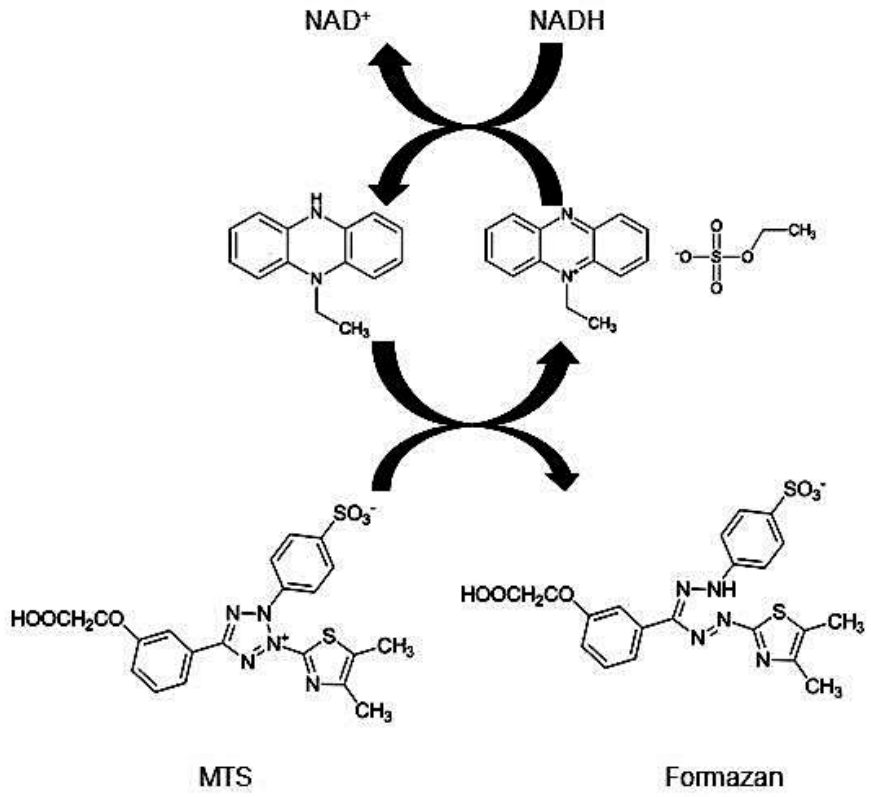


Figure 9. Reduction of MTS to Formazan (From Dessalegn E., 2015)

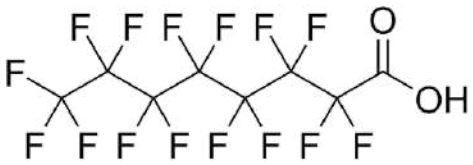
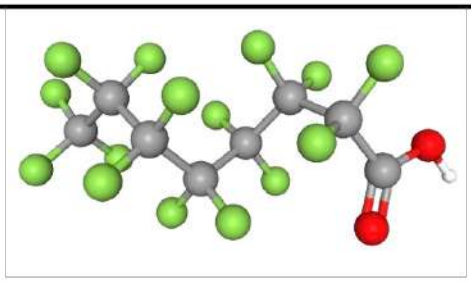
Because the MTS formazan product is directly soluble in the GM, the CellTiter 96® AQueous One Solution Assay eliminates a liquid handling step since a second addition of reagent to the assay plate is not needed to solubilize formazan precipitates, thus making the protocols more convenient when compared to MTT or INT assays.

2.1.4.1 Test Compounds

2.1.4.1.1 PFOA

PFOA (Table 7) also frequently referred to as C_8 and perfluorooctanoate, is a highly stable synthetic PFCA and fluorosurfactant. Used in several industrial applications, including carpeting, upholstery, apparel, floor wax, textiles, firefighting foam, sealants, as a surfactant in the emulsion in the emulsion polymerization of fluoropolymers and as a building block for the synthesis of *Alternative* PFAS polymers and polymeric materials.

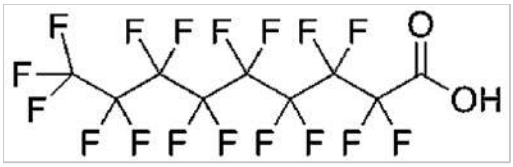
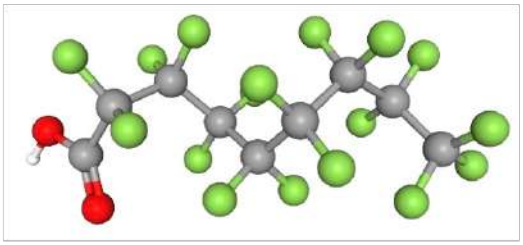
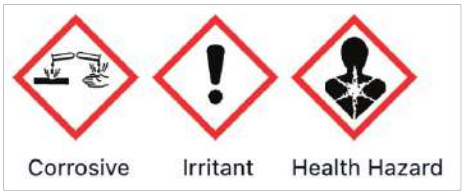
Table 7. Structures and chemical proprieties of Perfluorooctanoic Acid (PFOA).

Chemical Formula	$C_8HF_{15}O_2$
Physical Form	White or cream crystalline solid, with pungent odor.
2D Representation	
3D Structure	
Molecular Weight (g/mol)	414.7 g/mol
Melting Point (°C)	52 - 54 °C
Boiling Point (°C)	189 °C
Log P	6.3
Chemical Safety	

2.1.4.1.2 PFNA

PFNA (Table 8) is the largest synthetic PFCA and fluorosurfactant. Additionally, Amstutz et al. (2022) found, through the calculation of the IC_{50} values for the different PFAS, that the toxicity of the PFAS actually increased with the increase of the carbon chain length, with PFNA experimentally exhibiting the highest toxicity. (45) In acidic form it is a highly reactive strong acid. In its conjugate base form, commonly ion paired with ammonium, as a salt, it's stable. It's been considered a probable degradation product of many other compounds.

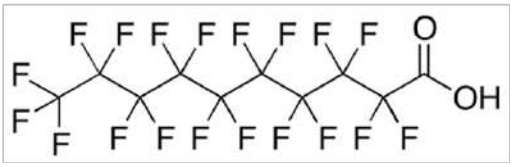
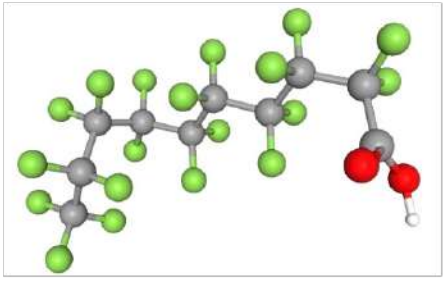
Table 8. Structures and chemical proprieties of Perfluorononan-1-oic Acid (PFNA).

Chemical Formula	$C_9HF_{17}O_2$
Physical Form	Beige crystalline solid.
2D Representation	
3D Structure	
Molecular Weight (g/mol)	464.08 g/mol
Melting Point (°C)	59 - 62 °C
Boiling Point (°C)	218 °C
Log P	5.6
Chemical Safety	

PFDA (Table 9) and PFUdA (Table 10), both PFAAs, are breakdown products of stain- and grease-proof coatings on food packaging, couches, and carpets. These substances have shown a role as xenobiotics and environmental contaminants, mechanistically related to an undecanoic acid. PFDA can also be used as a surfactant and flame retardant, however when heated to decomposition, it emits toxic vapors of F^-.

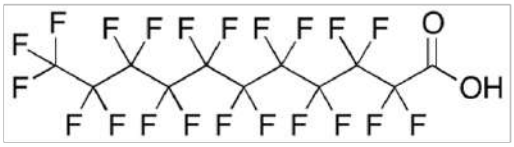
2.1.4.1.3 PFDA

Table 9. Structures and chemical proprieties of Perfluorodecanoic Acid (PFDA).

Chemical Formula	$C_{10}HF_{19}O_2$
Physical Form	May present as a white poder or a liquid.
2D Representation	
3D Structure	
Molecular Weight (g/mol)	514.08 g/mol
Melting Point (ºC)	77 - 81 °C
Boiling Point (ºC)	218 °C
Log P	6.3
Chemical Safety	

2.1.4.1.4 PFUdA

Table 10. Structures and chemical proprieties of Perfluoroundecanoic Acid (FFUdA).

Chemical Formula	$C_{11}HF_{21}O_2$
Physical Form	May present as a white poder or a liquid.
2D Representation	
Molecular Weight (g/mol)	564.09 g/mol
Log P	6.9
Chemical Safety	

**No 3D structure available.*

2.1.4.2 Controls

Negative controls (Table 12) are samples that undergo processing exactly the same as the others in the experiment but are not expected to change due to any variable in the experiment, while positive control (Table 11) samples will yield the predicted result.

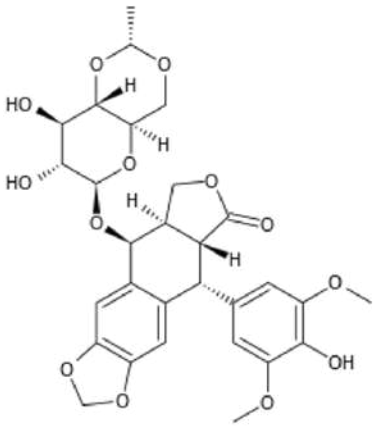
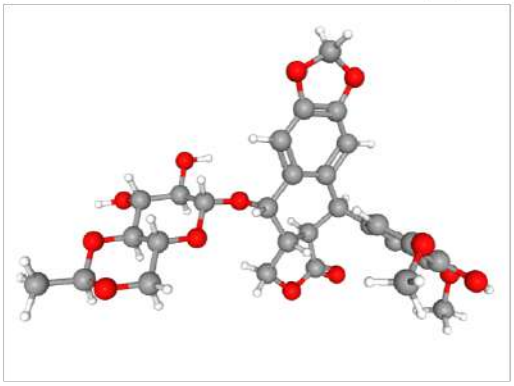
However, due to an error in the early phase of the procedure, a “Blank-well" was created, consisting only in a simple dilution of the cell suspension, which eventually made it possible to assess the inherent toxicity of DMSO itself.

2.1.4.2.1 ETO - Positive Control

Etoposide (ETO) is a beta-D-glucoside, a furonaphthodioxole and an organic heterotetracyclic compound, besides being a semisynthetic derivative of podophyllotoxin. ETO exhibits antitumor activity through a mechanism of action of topoisomerase inhibiting. The ETO-topoisomerase II-DNA complex inhibits DNA synthesis, inducing breaks in the DNA's double strand, while also hindering repair. Etoposide acts primarily in the G2 and S phases of the cell cycle, as accumulated damage in DNA prevents entry into the mitotic phase (M), resulting in cell death.

Using ETO as positive control can help ensure the validity and reliability of the study results.

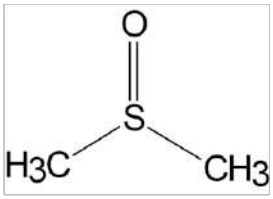
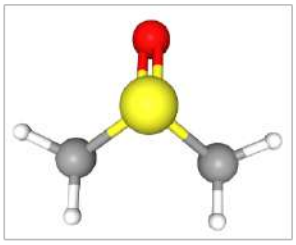
Table 11. Structures and chemical proprieties of Etoposide (ETO).

Chemical Formula	$C_{29}H_{32}O_{13}$
Physical Form	White to yellow-brown, crystalline powder.
2D Representation	**3D Representation**
	
Molecular Weight (g/mol)	588.6 g/mol
Log P	0.6
Chemical Safety	

2.1.4.2.2 DMSO 1% - Negative Control

Dimethyl sulfoxide (DMSO) is a highly polar organic reagent that has exceptional solvent properties for organic and inorganic chemicals. Is commonly used as a cryoprotectant because of its membrane penetrating and water displacement properties.

Table 12. Structures and chemical proprieties of Dimethyl sulfoxide (DMSO).

Chemical Formula	C_2H_6OS
Physical Form	Colorless liquid that is odorless or has a slight odor of sulfur, garlic, or oysters.
2D Representation	**3D Representation**
	
Molecular Weight (g/mol)	78.14 g/mol
Log P	-0.6
Chemical Safety	

Given that the cell culture used is not primary, a concentration of 1% should not result in significant toxicity for THP-1.

2.1.5 MTS Assay Protocol

The testing was carried out under the manufacturer's instructions.

1. Grow cells in RPMI-1640 Medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Sigma-Aldrich, USA) and 10% FBS (Gibco, Thermo Fisher Scientific, USA) at 37 °C and 5% CO_2;

2. Determine cell density and viability (by trypan blue exclusion) and resuspend the cells culture in two final concentrations, C_A=1.0×10^5 cells/mL and C_B=2.5x10^5 cells/mL, in the complete GM;

3. Dispense 50 µL of the cell suspension (respectively, 5000 and 12 500 cells per well) into 16 wells of the 96-well microplate;

4. Treat the cells with the test compounds (50 µL at a concentration of 100 µM), positive control (50 µM ETO) and negative control (0.5% DMSO);

   The total volume in each well should be 100 µL;

5. Incubate the plate for 72 hours in a humidified, 37°C and 5% CO_2 atmosphere;

6. Add 10 µL per well of MTT reagent (CellTiter 96® AQueous One Solution Reagent);

7. Incubate the plate for 4 hours in a humidified, 37°C and 5% CO_2 atmosphere;

8. Measure and record Absorbance at 490 nm resorting to the automatic 96-well microplate reader (Tecan Safire 2 Multi-Detection Plate Reader).

The cell suspensions, at intended cell densities, were prepared, when possible, after subculture of the THP-1 cell-line, for lineage maintenance purposes and continuity of the study. As the whole assay is based on a dilution of the solutions of test compounds, controls, and MTS reagent, in 50 µL of cell suspension, the following adaptation to the protocol was made, given the unavailability of a multichannel pipette – the conjunct volume of each solution needed for a triplicate run was prepared individual Eppendorf's of appropriated volume, so pipetting into the respective wells could also be optimized. (Annex A3.

3 Results and Discussion

Subculturing of THP-1 was done until a point of satisfactory stability of the cell line. Experiments began to be made around the fifth passage. The first two attempts at seeding an MTS microplate were discarded due to faulty execution cross-sectionally during the various attempts at the procedure from subcultures reaching far too high densities (>1,0x10^6 cells/mL), morphological anomalies, notably a reduction in size and abnormal shapes, even considering possible activation (A-THP-1), identified through microscopic observation; heterogenicity in the volume of the wells, due to leakage events and non-accurate pipetting; to accidental exposure to a concentration 3x higher than the pre-established, resulting in the complete unviability of the culture used to seed the microplate in question.

In Microplate #2, there was a protocol lapse whereby the *True Blank* wells were not seeded correctly after the protoculated 10µL of MTS reagent were added in, therefore making it impossible to quantify the interferences of the background, and subtract them from the Absorbance (Abs) results of that assay. Ultimately, an additional triplicate run, *Cells*, was created and subsequently repeated. The outcomes of this trial will allow evaluation of the actual extent of DMSO's toxicity on the cell culture, in addition to its impact and corroboration as positive control.

Therefore, for all the tests carried out, a total of eight 300µL solutions were prepared in duly labeled 0.5 mL Eppendorf's. 150 µL of THP-1 suspension, C_A and C_B, and 150 µL of 50 µM solution of PFOA, PFNA, PFDA, PFUdA, ETO, and 0.5% DMSO. As for the *Blank* wells Eppendorf, the 150 µL of cell suspension was diluted in 150 µL of GM. After approximately 4 hours, before measuring the Abs, the microplates and wells were observed macroscopically and microscopically, respectively. The records, results and observations made are detailed below solely for (some of the) viable cultures in which the MTS Assay was performed. Images of the subcultures deemed as non-viable are available for consultation in Annex A4.

All photographic records of the subcultures were done with a total magnification of 100×.

3.1.1 Subculture $P_{THP-1}7$

Subculture P7 ($P_{THP-1}7$) was used to seed this microplate. Population Doubling Time (PDT) was calculated (Annex A4.) for all the passages done, with the average PDT until this point being 42.42 hours. This is a rather reasonable rate, indicator of culture stability and thrive, given that the literature provided standard for this cell line ranges from 35 to 50 hours.

Table 13. Microplate #3 ($P_{THP-1}7$ at C_A e C_B) Absorbance (Abs) and Cell Viability (%) results.

MICROPLATE #3 (27/03) **T(exposure) ≈ 62h (<72h)**

	Abs			Average Abs	Cell Viability (%)		Abs			Average Abs	Cell Viability (%)
C_A=1,0x10^5 cells/mL											
PFOA	0,172	0,190	0,119	0,160	68,049	**ETO**	0,134	0,124	0,142	0,133	56,573
PFNA	0,178	0,292	0,163	0,211	89,387	**DMSO**	0,214	0,243	0,212	0,223	94,524
PFDA	0,168	0,194	0,158	0,173	73,397	**Cells**	0,243	0,276	0,188	0,236	100,000
PFUdA	0,216	0,214	0,221	0,217	92,005						
C_B=2,5x10^5 cells/mL											
PFOA	0,278	0,281	0,238	0,160	45,730	**ETO**	0,150	0,165	0,139	0,151	43,106
PFNA	0,298	0,297	0,258	0,211	60,070	**DMSO**	0,413	0,386	0,333	0,377	107,541
PFDA	0,255	0,267	0,239	0,173	49,325	**Cells**	0,341	0,368	0,342	0,351	100,000
PFUdA	0,401	0,400	0,325	0,217	61,830						

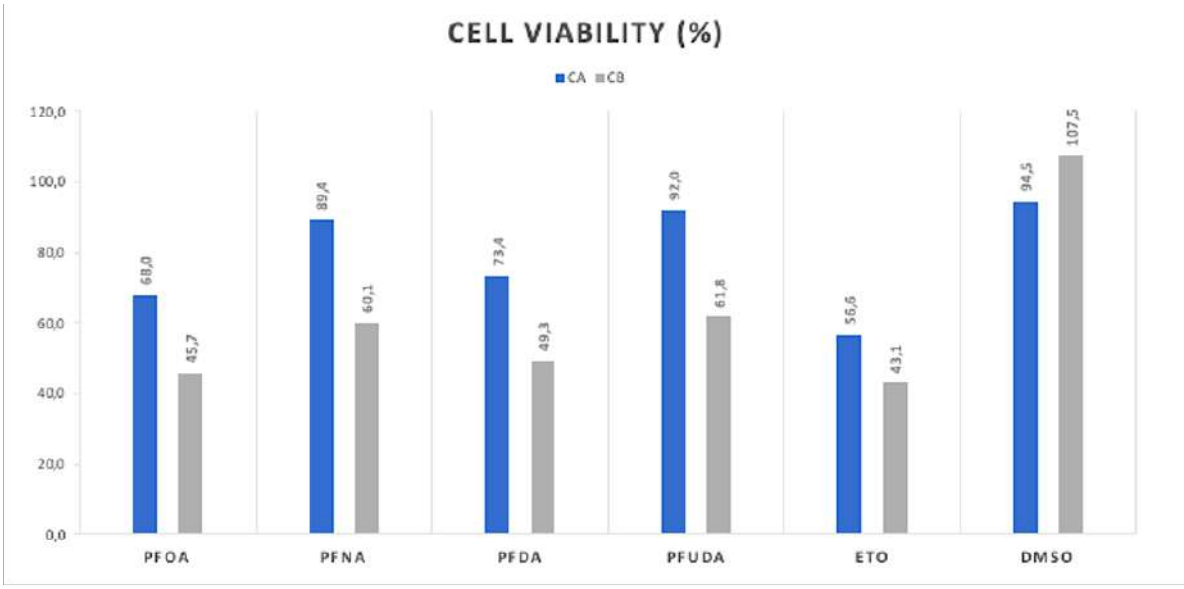


Figure 10. Microplate #3 ($P_{THP-1}7$ at C_A e C_B) Cell Viability (%) graph.

3.1.2 Subculture $P_{THP-1}8$

Microplate #4 was seeded with dilutions of PTHP-18. (PDT (PTHP-18) ≈ 31.1 h)

Table 14. Microplate #4 ($P_{THP-1}8$ at C_A e C_B) Absorbance (Abs) and Cell Viability (%) results.

MICROPLATE #4 (27/03→ 30/03) T(exposure) ≈ 60h (<72h)

	Abs			Average Abs	Cell Viability (%)		Abs			Average Abs	Cell Viability (%)
					C_A=1,0x10^5 cells/mL						
PFOA	0,103	0,166	0,150	0,140	61,640	**ETO**	0,191	0,194	0,050	0,145	63,859
PFNA	0,208	0,241	0,251	0,234	103,013	**DMSO**	0,194	0,254	0,191	0,213	93,827
PFDA	0,178	0,249	0,214	0,213	94,121	**Cells**	0,224	0,214	0,243	0,227	100,000
PFUdA	0,217	0,258	0,223	0,233	102,601	**BL**	0,070	0,073	0,069	0,070	
					C_B=2,5x10^5 cells/mL						
PFOA	0,315	0,366	0,340	0,340	75,999	**ETO**	0,141	0,110	0,118	0,123	27,443
PFNA	0,302	0,335	0,318	0,318	71,076	**DMSO**	0,442	0,523	0,414	0,459	102,568
PFDA	0,241	0,308	0,275	0,275	61,295	**Cells**	0,394	0,520	0,429	0,448	100,000
PFUdA	0,290	0,238	0,264	0,264	58,995	**BL**	0,080	0,056	0,070	0,069	

	Normalized Values					
	C_A			C_B		
	Average Abs	**Normalized Abs**	Cell Viability (%)	Average Abs	**Normalized Abs**	Cell Viability (%)
PFOA	0,140	0,069	44,397	0,340	0,272	71,655
PFNA	0,234	0,163	104,367	0,318	0,250	65,840
PFDA	0,213	0,143	91,478	0,275	0,206	54,290
PFUdA	0,233	0,162	103,771	0,264	0,196	51,573
ETO	0,145	0,075	47,614	0,123	0,054	14,311
DMSO	0,213	0,142	91,052	0,459	0,391	103,033
Cells	0,227	0,156	100,000	0,448	0,379	100,000

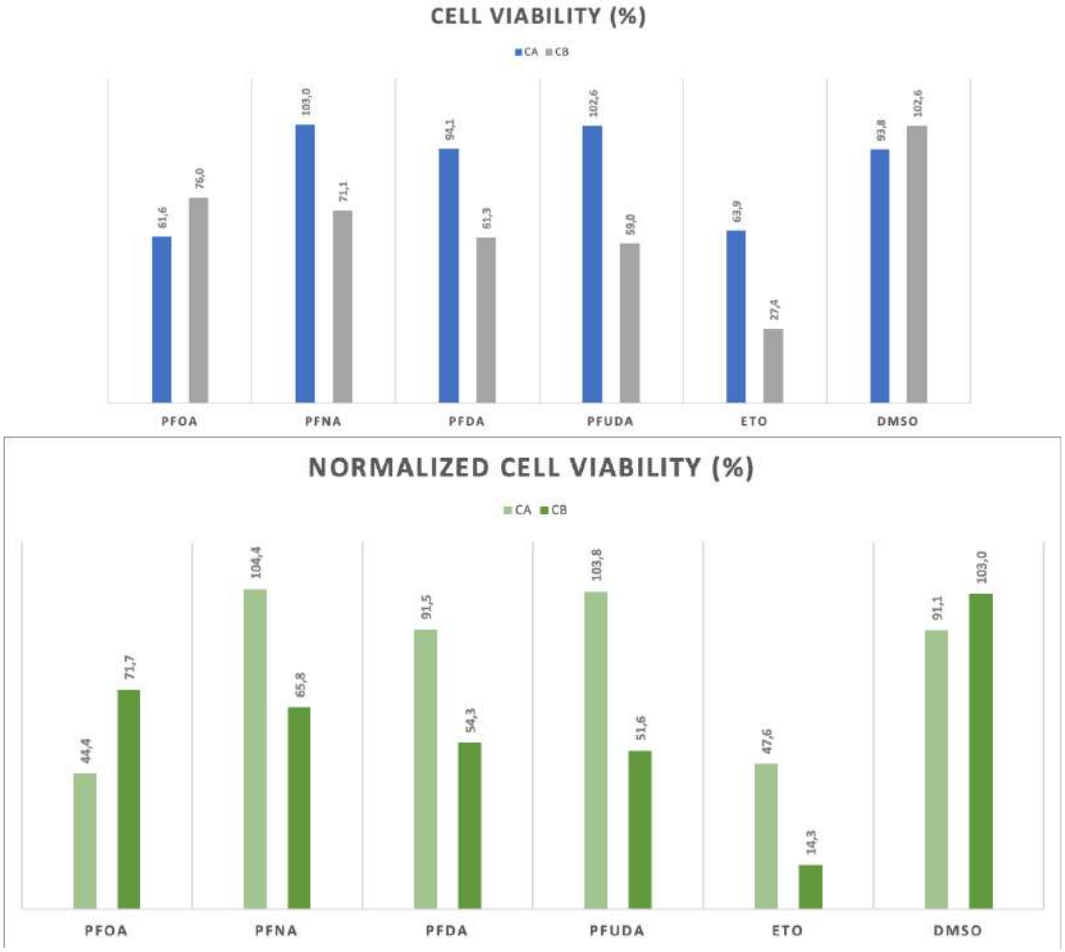


Figure 11. Microplate #4 ($P_{THP-1}8$ at C_A e C_B) Cell Viability (%) graphs.

It is relevant to recognize discrepancies in the volume of triplicate wells that might have occurred in this particular assay, namely those of exposure to PFOA, and treated with ETO. This might thus cast doubt on the data gathered from these wells and, in turn, any conclusions drawn on their toxicity regarding this particular microplate.

3.1.3 Subculture $P_{THP\text{-}1}10$

The third viable microplate on which the MTS-assay was thoroughly performed was Microplate #5. This microplate was seeded with $P_{THP\text{-}1}10$ (PDT ($P_{THP\text{-}1}9$) = 38 h → PDT ($P_{THP\text{-}1}10$) ≈ 32.34 h).

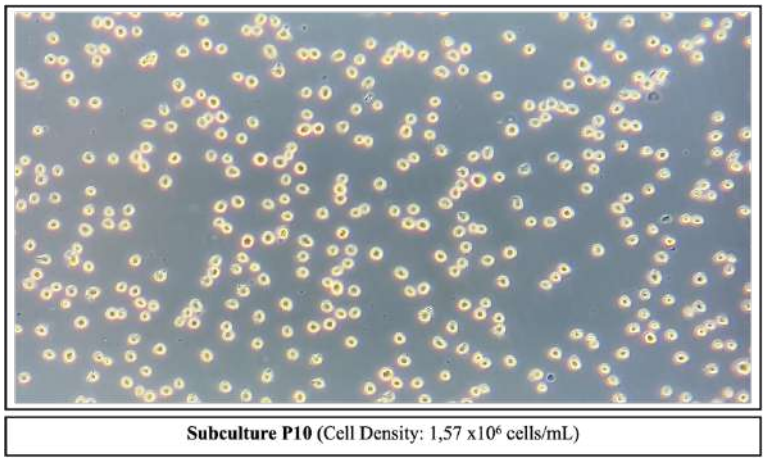
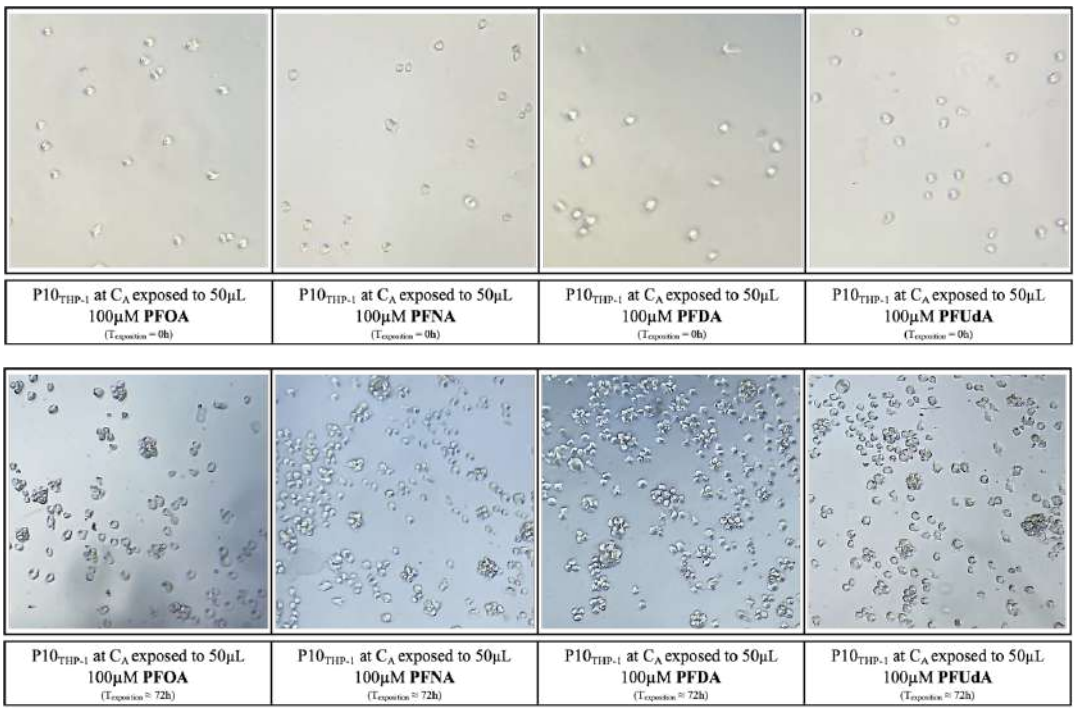


Figure 12. Photographic record of $P_{THP\text{-}1}10$ prior to passage and execution of MTS Assay.



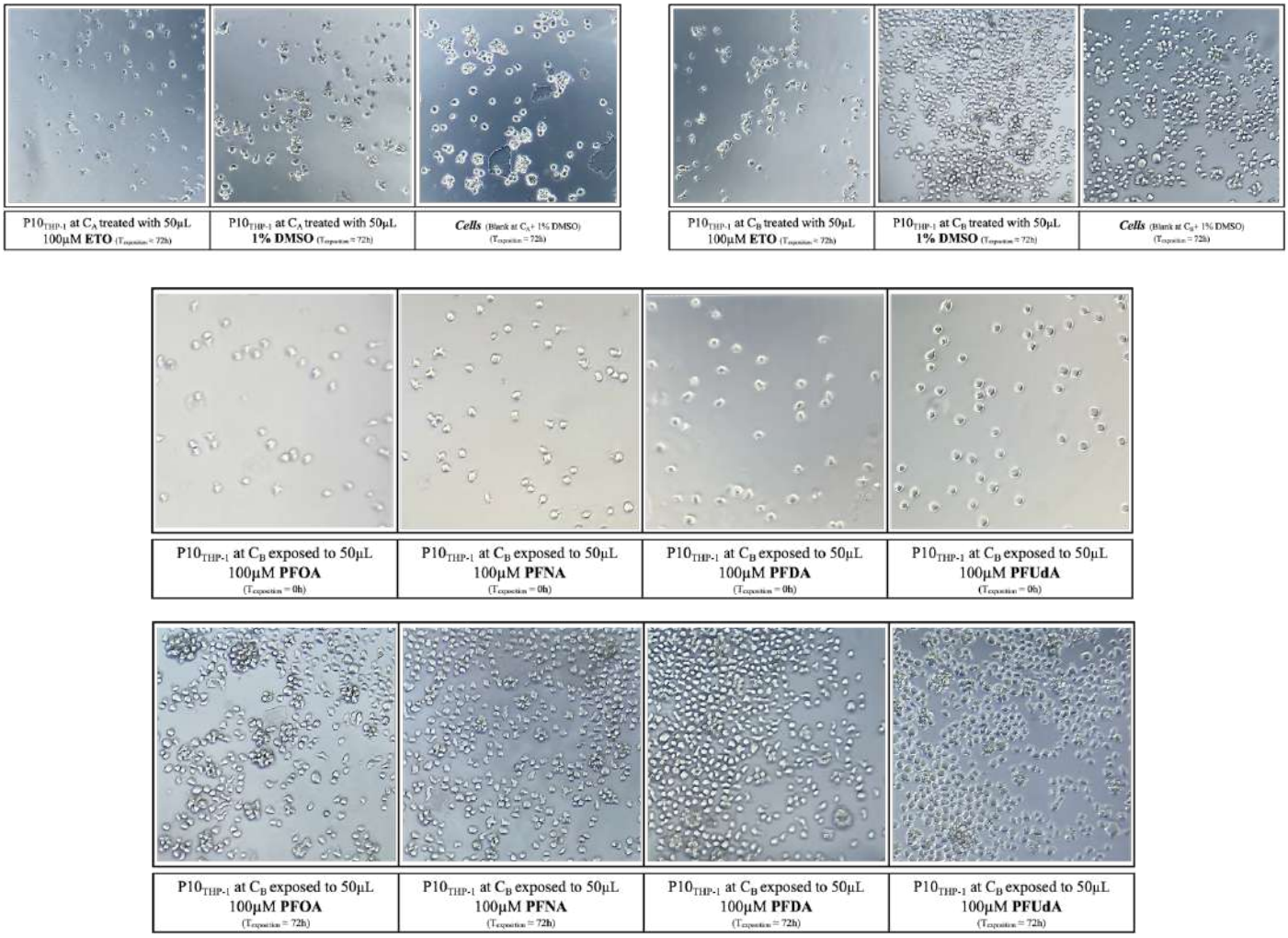


Figure 13. Photographic record of $P_{THP\text{-}1}10$, at CA (above) and CB (below), before and after exposure.

Table 15. Microplate #5 ($P_{THP\text{-}1}10$ at C_A e C_B) Absorbance (Abs) and Cell Viability (%) results.

MICROPLATE #5 (03/04→ 06/04) **T(exposure) ≈ 72h**

	Abs			Average Abs	Cell Viability (%)		Abs			Average Abs	Cell Viability (%)
					CA=1,0x10^5 cells/mL						
PFOA	0,1371	0,1445	0,1098	0,130	87,503	**ETO**	0,0902	0,0901	0,0936	0,091	61,234
PFNA	0,1331	0,1375	0,1373	0,136	91,192	**DMSO**	0,1388	0,1474	0,1293	0,139	92,891
PFDA	0,1345	0,1341	0,1246	0,131	87,905	**Cells**	0,1408	0,1739	0,1326	0,149	100,000
PFUdA	0,0703	0,0733	0,0699	0,071	47,731	**BL**	0,072	0,0742	0,0676	0,071	
					CB=2,5x10^5 cells/mL						
PFOA	0,2065	0,2014	0,1839	0,197	102,761	**ETO**	0,0911	0,0941	0,0904	0,092	47,856
PFNA	0,2672	0,4367	0,3457	0,350	182,254	**DMSO**	0,1895	0,2281	0,1911	0,203	105,695
PFDA	0,1993	0,2055	0,1824	0,196	101,962	**Cells**	0,1957	0,214	0,1662	0,192	100,000
PFUdA	0,214	0,2016	0,1694	0,195	101,580	**BL**	0,0751	0,0748	0,0652	0,072	

	Normalized Values					
	CA			CB		
	Average Abs	**Normalized Abs**	Cell Viability (%)	Average Abs	**Normalized Abs**	Cell Viability (%)
PFOA	0,130	0,059	76,060	0,197	0,126	104,407
PFNA	0,136	0,065	83,126	0,350	0,278	231,292
PFDA	0,131	0,060	76,831	0,196	0,124	103,132
PFUdA	0,071	0,000	-0,128	0,195	0,123	102,522
ETO	0,091	0,020	25,739	0,092	0,020	16,768
DMSO	0,139	0,067	86,381	0,203	0,131	109,091
Cells	0,149	0,078	100,000	0,192	0,120	100,000

This microplate revealed to be somewhat problematic to interpret and transpose into the overall results, since a leakage event was indeed confirmed in several wells, the most blatant being the triplicate PFUdA. Reinforcing the point made earlier that occurrences such as those described influence the results obtained and have been taken into account when making the final elations.

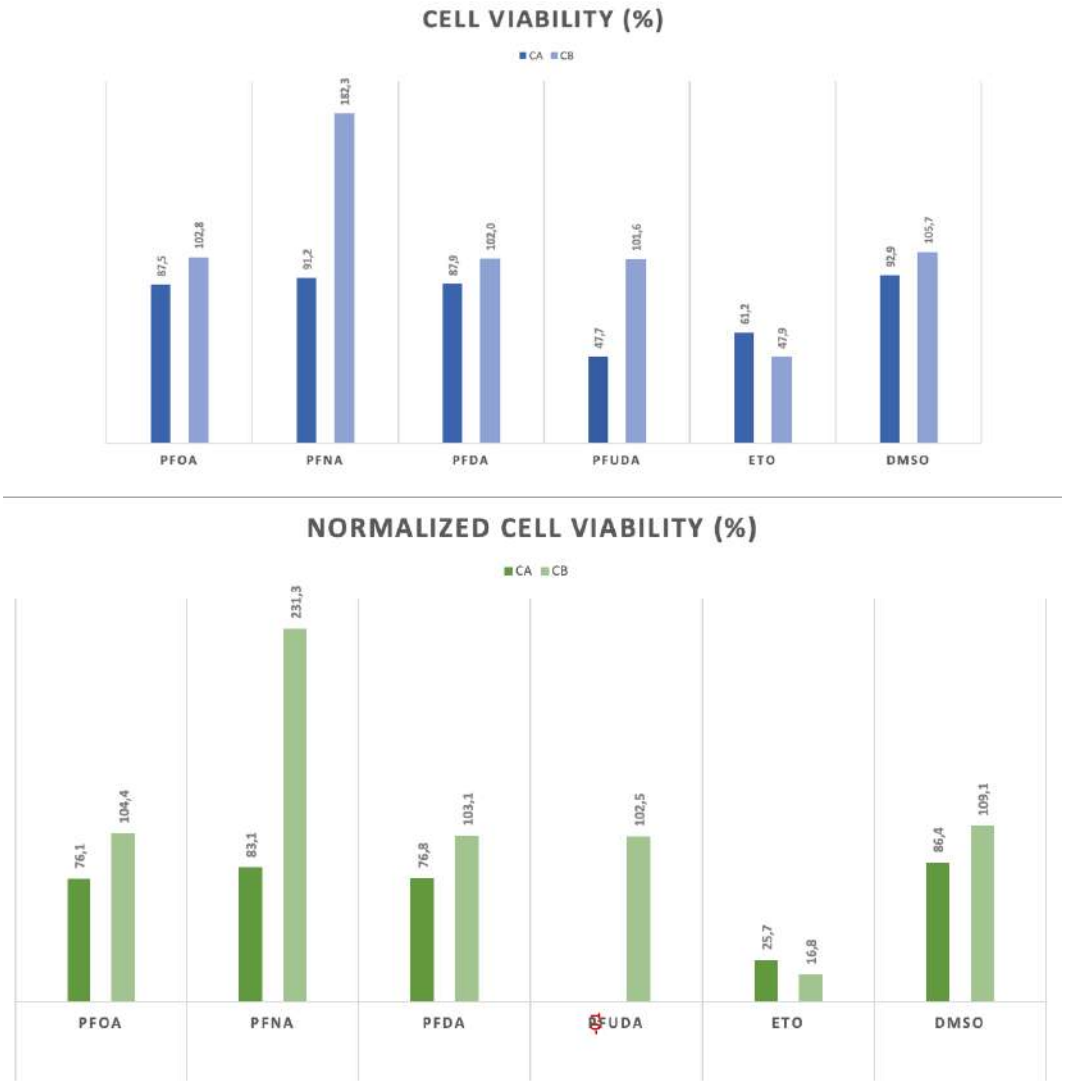


Figure 14. Microplate #5 ($P_{THP\text{-}1}10$ at C_A e C_B) Cell Viability (%) graphs.

Also, worth noting that even though $P_{THP\text{-}1}10$ exceeded slightly the recommended density range, with a cell density of 1.57 x10^6 cells/mL, the culture visually seemed healthy and exhibited a PDT ($P_{THP\text{-}1}10$) ≈ 32.34h. In the subsequent passage, the dilution performed was slightly larger to balance a potential transitory phase of increased activity and cell division.

3.1.4 Subculture $P_{THP\text{-}1}11$

$P_{THP\text{-}1}10$ was subcultured, giving rise to $P_{THP\text{-}1}11$. The generally good condition of this subculture was supported, besides a satisfactory macro and microscopic appearance, by a PDT ($P_{THP\text{-}1}11$) ≈ 37,45h.

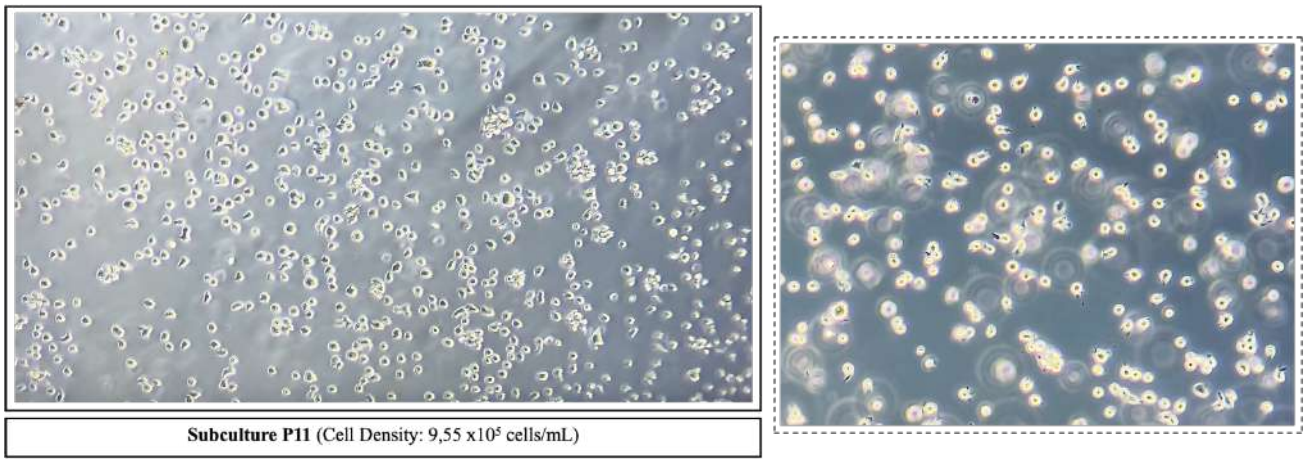


Figure 15. Photographic record of $P_{THP\text{-}1}11$ prior to passage and MTS Assay.

A more in-depth examination of the culture drew attention to the slightly altered cell morphology, which was the closest to the characteristic morphology of A-THP-1, described by Tominaga *et al.* (1998), and already reviewed in this paper. Furthermore, the culture appeared to be slightly adhered to the culture vessel, which is also characteristic of monocytic strains when they undergo activation.

$P_{THP\text{-}1}11$ was also subcultured, and $P_{THP\text{-}1}12$ however, all of the records and results obtained from this subculture appear to indicate that there was some kind of incubation problem, since, unlike the previous subculture, there was very little cell growth (only reaching a cell density of $2.5x10^5$ cells/mL), with a PDT ($P_{THP\text{-}1}12$) ≈ 97.7 hours, as well as an abnormally altered cell morphology, with generally smaller cells.

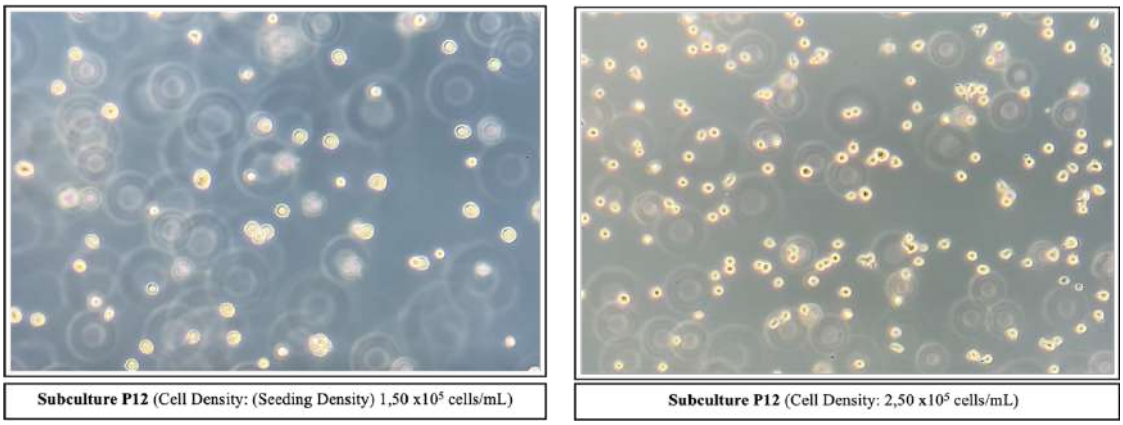


Figure 16. $P_{THP\text{-}1}12$ at C_A (right) and C_B (left).

Table 16. Microplate #6 ($P_{THP\text{-}1}12$ at C_A e C_B) Absorbance (Abs) and Cell Viability (%) results.

MICROPLATE #6 (11/04→ 14/04) **T(exposure) ≈ 67h (<72h)**

	Abs			Average Abs	Cell Viability (%)		Abs			Average Abs	Cell Viability (%)
					C_A=1,0x10^5 cells/mL						
PFOA	0,1882	0,1943	0,1812	0,188	83,834	**ETO**	0,1861	0,1774	0,2133	0,192	85,782
PFNA	0,1461	0,1622	0,1826	0,164	73,007	**DMSO**	0,2142	0,2297	0,2172	0,220	98,319
PFDA	0,1364	0,1487	0,1388	0,141	63,043	**Cells**	0,2276	0,2305	0,2143	0,224	100,000
PFUdA	0,1437	0,1527	0,1506	0,149	66,478	**BL**	0,0706	0,0692	0,069	0,070	
					C_B=2,5x10^5 cells/mL						
PFOA	0,3327	0,3098	0,3238	0,322	98,592	**ETO**	0,2059	0,2315	0,2377	0,225	68,881
PFNA	0,2835	0,3168	0,3049	0,302	92,358	**DMSO**	0,2781	0,3315	0,3582	0,323	98,745
PFDA	0,1318	0,1356	0,1368	0,135	41,241	**Cells**	0,3219	0,3373	0,3209	0,327	100,000
PFUdA	0,1346	0,1571	0,1394	0,144	43,985	**BL**	0,0719	0,0696	0,0713	0,071	

	Normalized Values					
	C_A			C_B		
	Average Abs	**Normalized Abs**	Cell Viability (%)	Average Abs	**Normalized Abs**	Cell Viability (%)
PFOA	0,188	0,118	76,553	0,322	0,251	98,201
PFNA	0,164	0,094	60,850	0,302	0,231	90,238
PFDA	0,141	0,072	46,398	0,135	0,064	24,945
PFUdA	0,149	0,079	51,381	0,144	0,073	28,450
ETO	0,192	0,123	79,379	0,225	0,154	60,250
DMSO	0,220	0,151	97,563	0,323	0,252	98,397
Cells	0,224	0,155	100,000	0,327	0,256	100,000

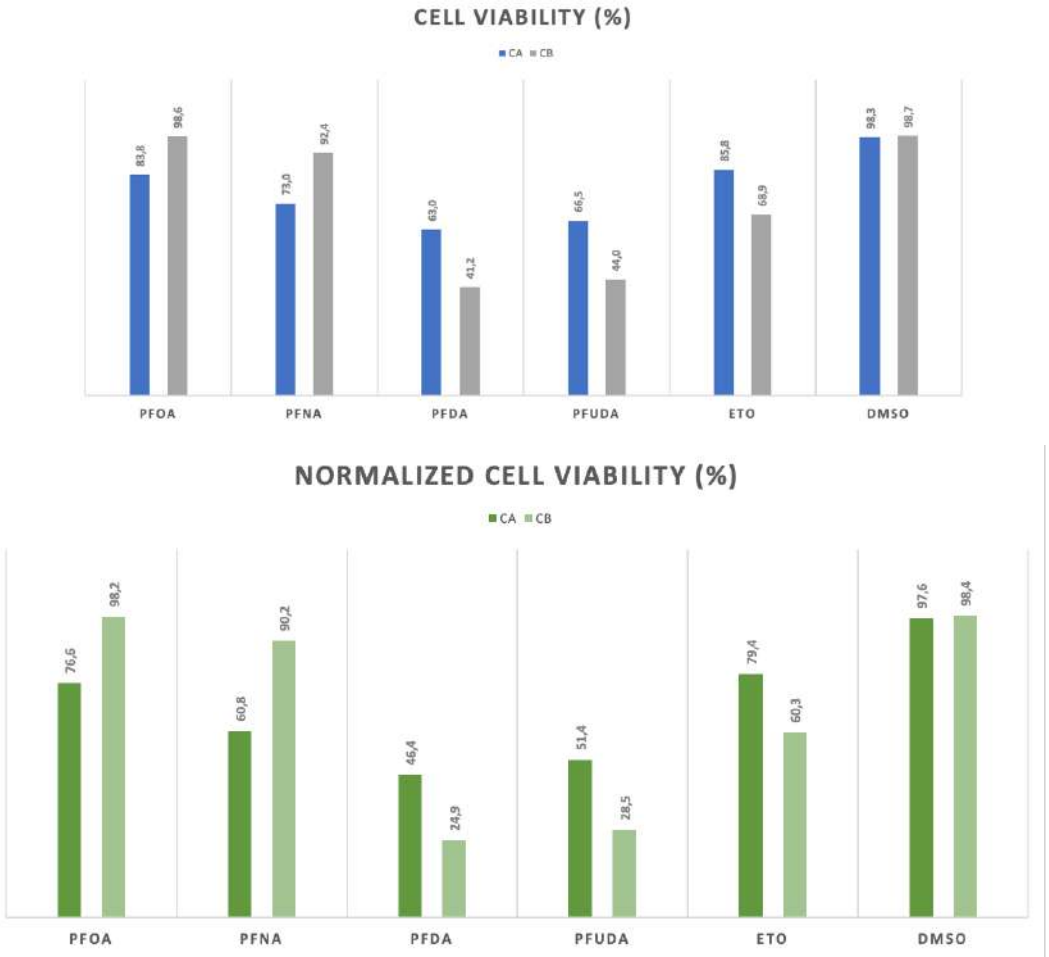


Figure 17. Microplate #6 ($P_{THP\text{-}1}12$ at C_A e C_B) Cell Viability (%) graphs.

The average period of exposure to PFAS was approximately 65,25 hours, with the difference between this and the pre-defined exposure time of 72 hours being considered non-significant when it came to overall findings. Available research data was used as a basis for such conclusions, considering ETO was shown not to influence M1 or M2 polarization. (116); studies where THP-1-derived macrophages were treated with PFOS in a concentration-dependent manner, cytosolic DNA was able to be extracted (110); other studies have shown that PFOA and PFOS suppressed LPS-induced TNF-α production in primary human cultures and THP-1 cells. (109) Additionally, PFAS structures can resemble fatty acids and behave similarly to endogenous substrates by catalyzing differentiation of THP-1 monoblasts, and activation as macrophages. (120) Overall, even though it's not straightforward to link in some way such events with THP-1 activation, this phenomenon, which was verified before, and more plausibly after, exposure to the PFAS test compounds, may be highlighted, with some studies suggesting that PFAS can activate the innate immune system through the AIM2 inflammasome. (110) Once again, PFAS fatty-acid-like structures allows them to behave similarly to endogenous substrates by catalyzing differentiation of THP-1 monoblasts into macrophages, and subsequent activation. (121) Another valid hypothesis may consist in the continuous activation of PPARγ via its agonists, which was also shown to promote activation in Bone Marrow-Derived Macrophages (BMDM) and THP-1 cells. (20) Furthermore, PFOS treatment could enhance caspase-3 and Poly (ADP-ribose) polymerase (PARP) cleavage, in both THP-1-derived macrophages and BMDMs. (110) Still, this remain theoretical conjectures, as the conducted research does not enable such an exhaustive understanding of the underlying toxicological mechanics.

4 Conclusions & Future Research Prospectives

Human exposure to PFAS has been connected to several adverse health outcomes and, as a result, to a diversity of clinical patterns of disease. To better portray the underlying mechanisms of toxicity, animal studies have been conducted, although these may be hindered by human and most animal models (mainly rodents) inherent metabolic differences. Solid knowledge that allows for a cross-sectional understanding of the toxicological effects should thus be obtained through integration of animal research data and *in vitro* evidence from human cell-based models.

Most of the evidence on immunotoxicity resulting from PFAS exposure refers to the representative or *Legacy* PFAS - PFOA and PFOS - with a particular focus on the PFAAs sub-family. Most people in developed countries have detectable serum concentrations of PFOA ranging from 1 to 10 ppb, while reported PFOS serum concentrations in general populations are somewhat higher than those for PFOA. Nevertheless, is also worth noting that even so the range of exposure concentrations primarily explored is often significantly greater than the one verified in typical exposure settings, with worries regarding the impact of chronic cumulative exposure to PFAS mixtures beginning to emerge. Similarly, many of the topics discussed in this review warrant further investigation, as study findings are still limited and/or inconsistent. Studies within the same subsection reporting different findings for the same biological outcome may be due to inconsistent levels of exposure being examined and compared across studies or may be due to a lack of consideration of important variables such as age, parity, or race. All of these variables are now being taken into account and steering current PFAS research trends.

In summary, *in vitro*, *in vivo,* and epidemiologic studies on PFAS inherent toxicity have been informative, but unfortunately not entirely conclusive yet. Each study design has strengths and limitations that need to be carefully considered when interpreting study findings. Weaknesses in the study design can lead to questionable associations or make it more difficult to detect true ones if they are present. Knowing that no research will be entirely shielded from such weaknesses, an intersectional approach, that incorporates data from a variety of studies, might reveal beneficial in drawing causal associations, ideally regarding a subgroup or even the PFAS family, while being supported by different kinds and degrees of evidence.

Regarding the cell viability assay (MTS Assay) carried out on THP-1 cells (at two pre-establishes cell densities (C_A and C_B)) exposed to PFAS - PFOA, PFNA, PFDA and PFUdA - could begin to be interpreted through the already proven impact of PFAS on monocytes, WBCs that constitute a critical component of the innate immune system, playing a role in both the inflammatory and anti-inflammatory processes that take place during an immune response. Studies have shown that PFAS exposure can lead to suppressed production of cytokines by monocytes, and reduced phagocytosis by macrophages, leading to immune dysregulation and increased immunosusceptibility. The molecular mechanisms underlying such immune effects of PFAS on monocytes are not yet fully understood.

Table 17. Average overall Cell Viability (%) results at C_A (above) and C_B (below).

Compound	C_A=1,0x10^5 cells/mL				
	Cell Viability (%)				Average Cell Viability (%)
	MICROPLATE #3	MICROPLATE #4	MICROPLATE #5	MICROPLATE #6	
PFOA	68,049	61,640	87,503	83,834	75,256
PFNA	89,387	103,013	91,192	73,007	89,150
PFDA	73,397	94,121	87,905	63,043	79,617
PFUdA	92,005	102,601	47,731	66,478	77,204
ETO	56,573	63,859	61,234	85,782	66,862
DMSO	94,524	93,827	92,891	98,319	94,890

C_A	Cell Viability (%)
PFOA	75,3
PFNA	89,1
PFDA	79,6
PFUdA	77,2
ETO	66,9
DMSO	94,9
Desvio Padrão	10,07003916

Compound	C_B=2,5x10^5 cells/mL				
	Cell Viability (%)				Average Cell Viability (%)
	MICROPLATE #3	MICROPLATE #4	MICROPLATE #5	MICROPLATE #6	
PFOA	45,730	75,999	102,761	98,592	80,771
PFNA	60,070	71,076	182,254	92,358	101,439
PFDA	49,325	61,295	101,962	41,241	63,456
PFUdA	61,830	58,995	101,580	43,985	66,598
ETO	43,106	27,443	47,856	68,881	46,821
DMSO	107,541	102,568	105,695	98,745	103,637

C_B	Cell Viability (%)
PFOA	80,8
PFNA	101,4
PFDA	63,5
PFUdA	66,6
ETO	46,8
DMSO	103,6
Desvio Padrão	22,46497343

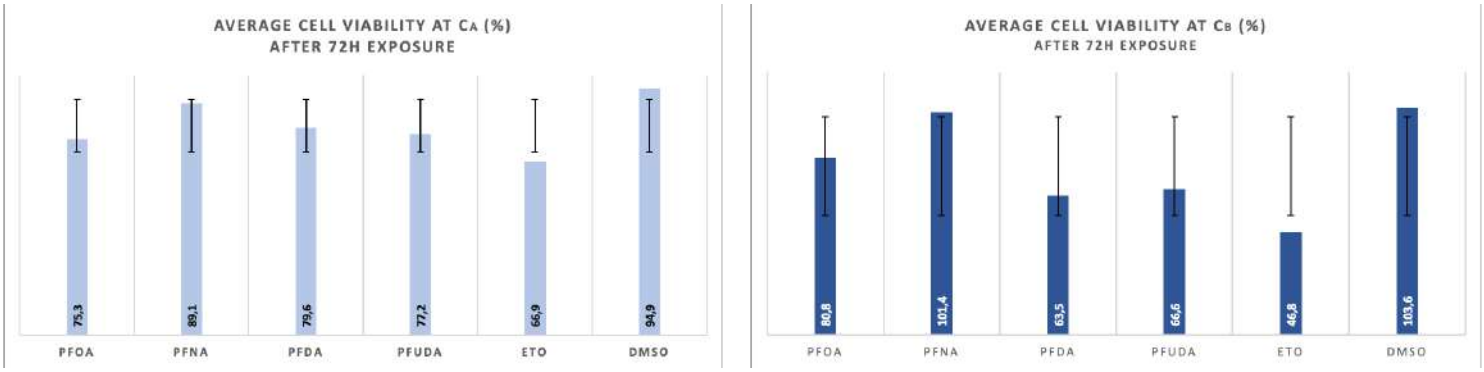


Figure 18. Comparative look on Cell Viability (%) at C_A and C_B.

	Cell Viability (%)	
	C_A	C_B
PFOA	75,3	80,8
PFNA	89,1	101,4
PFDA	79,6	63,5
PFUdA	77,2	66,6
ETO	66,9	46,8
DMSO	94,9	103,6

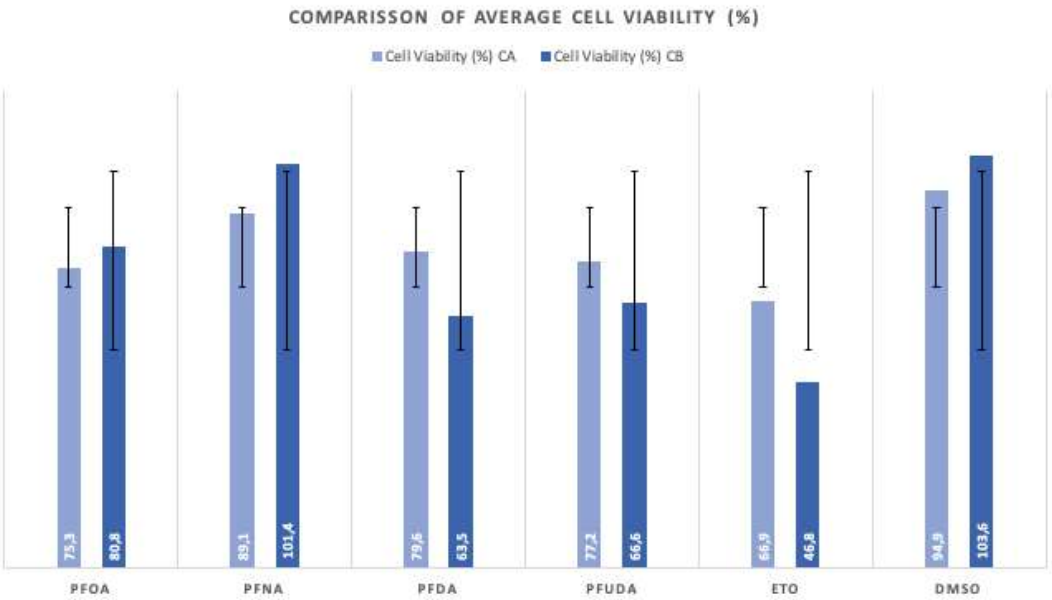


Figure 19. Graphs of average overall Cell Viability (%) at C_A (left) and C_B (right).

As a result of a protocol lapse in the first run, it was impossible to determine background interference that specific microplate. For this reason, a new data set was processed, excluding Microplate #2, to obtain normalized the results.

Table 18. Normalized average overall Cell Viability (%) results at C_A (above) and C_B (below).

Compound	C_A=1,0x10^5 cells/mL				
	Normalized Cell Viability (%)				Average Cell Viability (%)
	MICROPLATE #3	MICROPLATE #4	MICROPLATE #5	MICROPLATE #6	
PFOA		44,397	76,060	76,553	65,670
PFNA		104,367	83,126	60,850	82,781
PFDA		91,478	76,831	46,398	71,569
PFUdA		103,771	-0,128	51,381	51,674
ETO		47,614	25,739	79,379	50,911
DMSO		91,052	86,381	97,563	91,665

C_A	Cell Viability (%)
PFOA	65,7
PFNA	82,8
PFDA	71,6
PFUdA	51,7
ETO	50,9
DMSO	91,7
Desvio Padrão	16,42392715

Compound	C_B=2,5x10^5 cells/mL				
	Normalized Cell Viability (%)				Average Cell Viability (%)
	MICROPLATE #3	MICROPLATE #4	MICROPLATE #5	MICROPLATE #6	
PFOA		71,655	104,407	98,201	91,421
PFNA		65,840	231,292	90,238	129,123
PFDA		54,290	103,132	24,945	60,789
PFUdA		51,573	102,522	28,450	60,849
ETO		14,311	16,768	60,250	30,443
DMSO		103,033	109,091	98,397	103,507

C_B	Cell Viability (%)
PFOA	91,4
PFNA	129,1
PFDA	60,8
PFUdA	60,8
ETO	30,4
DMSO	103,5
Desvio Padrão	35,45523465

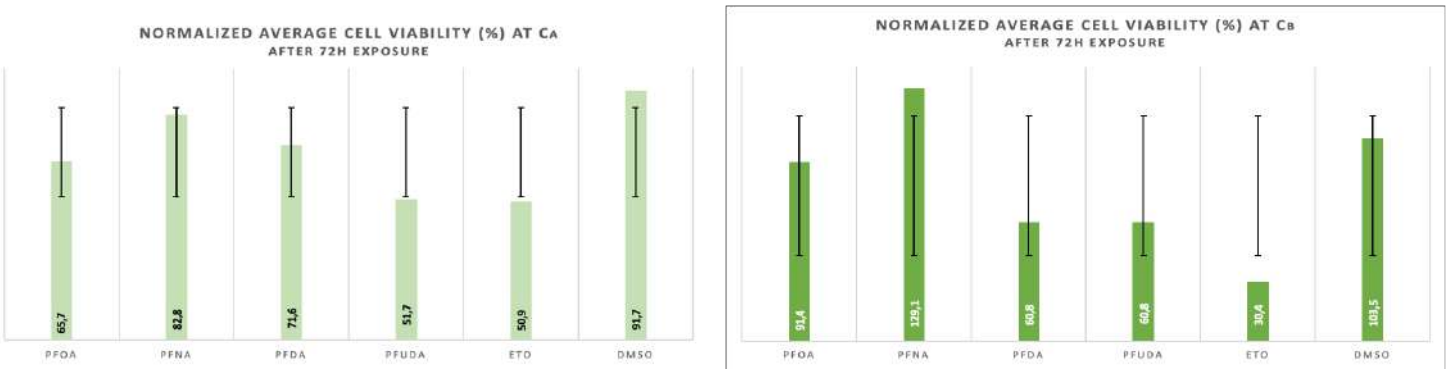
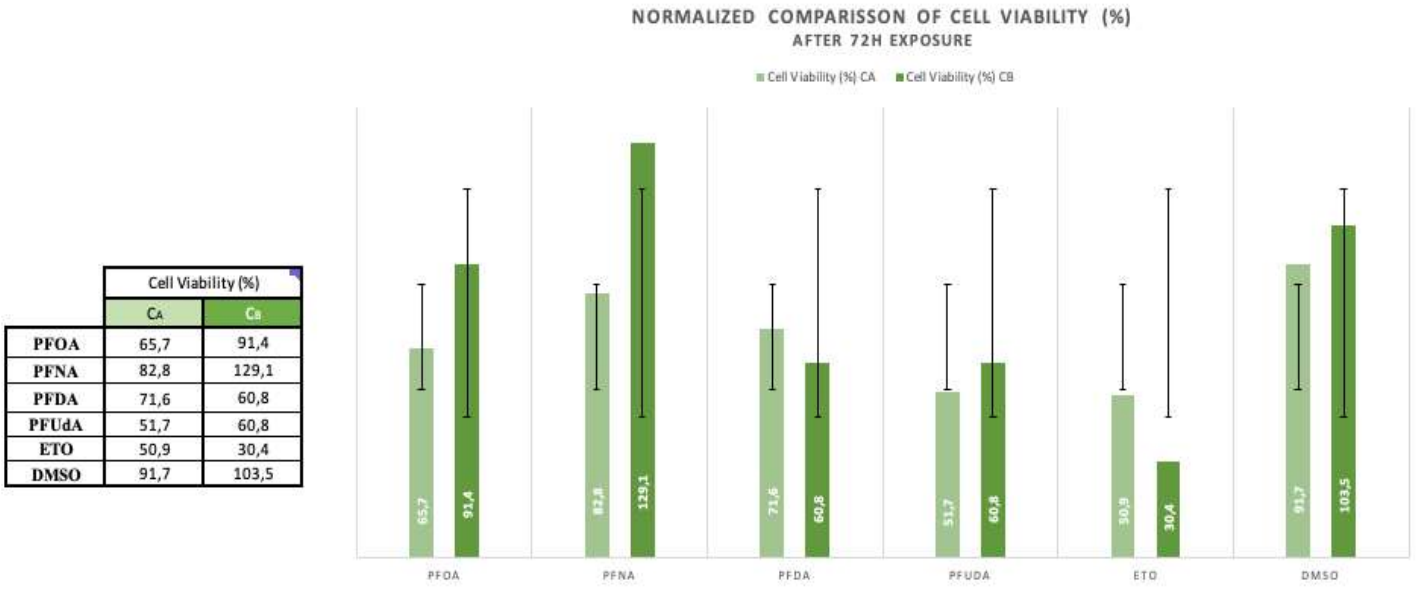


Figure 21. Graphs of normalized average overall Cell Viability (%) at C_A (left) and C_B (right).



	Cell Viability (%)	
	CA	CB
PFOA	65,7	91,4
PFNA	82,8	129,1
PFDA	71,6	60,8
PFUdA	51,7	60,8
ETO	50,9	30,4
DMSO	91,7	103,5

Figure 20. Comparative look on Normalized Cell Viability (%), at C_A and C_B.

Background interference was deemed not to be significant, and both sets of processed data could be taken into account when drawing conclusions from the field work developed. With that stated was possible to verify that, despite the compounds' physical and chemical resemblance, with similar molecular weights and even the same Topological Polar Surface Area (TPSA), 37.3 Å², there was a proportional rise in toxicity with increase of the carbon chain length and degree of fluorination. Compounds with a shorter chain length and lower degree of fluorination, PFOA and PFNA, more widely and extensively studied than their counterparts, demonstrated greater toxicity at the lowest cell density tested ($C_A = 1{,}0x10^5$ cells/mL), whereas PFDA and PFUdA, with slightly longer chain lengths and fluorination, demonstrated comparatively greater toxicity at the highest cell density tested ($C_B = 2{,}5x10^5$ cells/mL). These findings reinforce and corroborate the maxim that PFAS toxicity pathways are compound-specific. It was also possible to validate the use of DMSO as a negative control, since the overall

average viability of THP-1 cells treated with the organosulfur compound was roughly 100% overall (≈ 97.6%).

Regarding the chosen cell lineage, THP-1, advantages and drawbacks linked to the use might be raised. Its homogeneous genetic background and well-characterized nature, which minimizes the degree of variability in the cell phenotype; the straightforwardness of culture and passage processes; the capacity for polarization into M1 or M2 THP-1 macrophages, in response to cytokines or other *stimuli*, and the possibility of genetically modification, which can be a significant advantage when investigating the role of specific proteins in the immunotoxicity pathways of PFAS.

However, factors such as the lack of known mechanism(s) of immunotoxicity; different response data compared to primary human cultures, which makes it impossible, through THP-1, culture derived from a tumor, to fully characterize the response of the human immune system to PFAS, as one cannot simply assume that the phenotypic and molecular attributes of the macrophages derived from their differentiation are necessarily equivalent to those of macrophages obtained from circulating monocytes; how THP-1 cells differentiation requires induction by phorbol ester (PMA) or granulocyte-macrophage colony-stimulating factor (GM-CSF), which may not fully represent the response of macrophages *in vivo*; and finally how THP-1 cells are mainly used as a simplified model of human macrophages when investigating relatively straightforward biological processes, such as polarization and its functional implications, but not as an alternative source in more comprehensive immunopharmacology and drug screening programs; can all be raised as limitations to such studies, and should always be considered when interpreting the results.

Nevertheless, it seems that THP-1 lineage might be the most appropriate cell line for further research aiming to bridge the current knowledge gap in monocyte exposure within the setting of WBCs exposure and overall immunotoxicity.

In terms of protocol optimization suggestions and future research prompts, the recommendation for higher cell densities, such as 4.5–5.0x10^5 cells/mL (450–500 cells/mL), translates to a more average value within the typical range of circulating monocytes. Additional Abs measurements would also help strengthen the global toxicity scenario. Variations of the protocol might potentially be implemented, including co-culturing THP-1, which provides several advantages when studying PFAS-immunotoxicity pathways, namely, better mimic of *in*

vivo conditions, enabling a induced and/or comparative scenario and analysis; or even exposing the culture to a mixture of PFAS.

Executional optimizations may involve the use of a multichannel pipette, in order to reduce heterogenicity in the volume of the different wells, most importantly between wells of the same triplicate; incubation of the THP-1 cell suspension in the assay microplate for 24 hours before exposing it to the test compounds and subsequently to the MTS reagent, given that, even THP-1 being a suspension culture, a more preventive approach to cellular stress contexts would be beneficial and protective of pre-exposure cell viability; or even seek to better stabilize the cell line before starting experiments. This last suggestion requires caution as genetic drift might happen, i.e., a cell line cultured at high passage numbers, or for prolonged times, can show chromosomal duplications or rearrangements, mutations, and epigenetic changes Consequently, morphology, proliferation rate, metabolic capacity, or general cell health can change dramatically, affecting experimental outcomes. Lastly, calculation of half maximal inhibitory concentration (IC_{50}) would also be recommended since it would add valuable information to this cell viability experiment, focused on PFAS-induced immunotoxicity in THP-1 cells, this being dose-response relationship, comparative analysis, mechanistic insights, standardization of the experimental conditions and insurance of reproducibility, and optimization.

Overall, it should be emphasized that the prevailing opinion of experts on PFAS toxicity was that each PFAS needs to be evaluated individually and that extrapolation of data from PFOA and PFOS to other PFAAs, or even the whole PFAS family is not supported at the present.

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Annexes

A1. Molecular interaction between PFAS-PPAR.

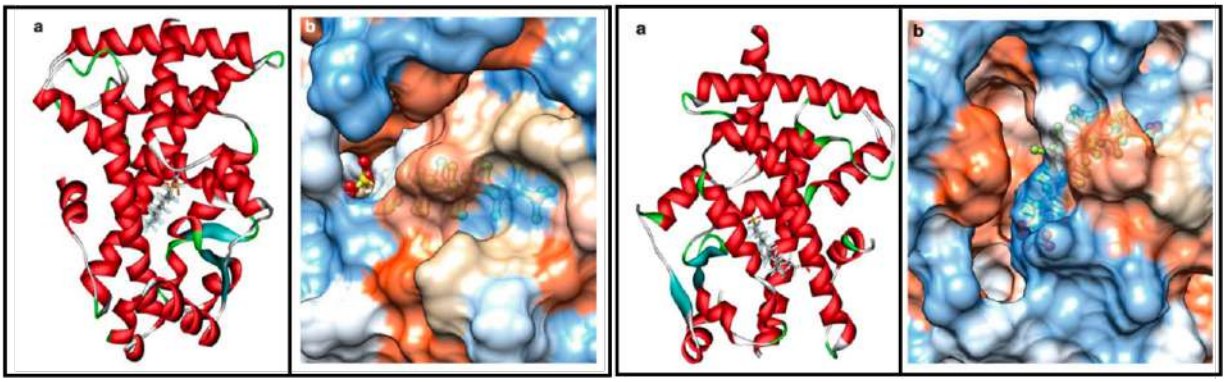


Figure 22. Ribbon (a) and surface (b) representation of PFAS in the structure of PPARα (right) PPARγ (left). (Adapted from: DeWitt, J. C. (Ed.), 2015)

A2. Molecular interaction between PFAS-ERα .

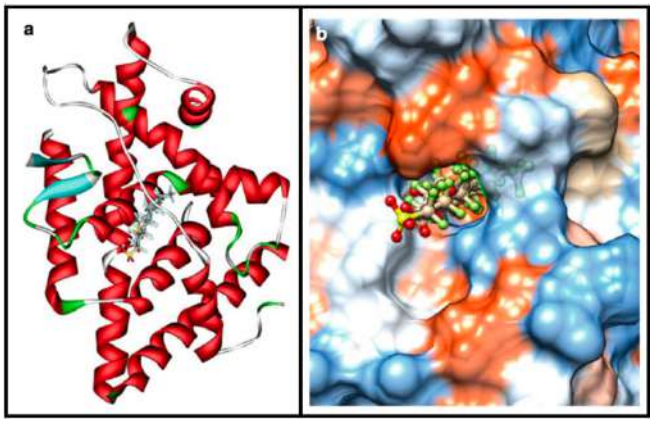
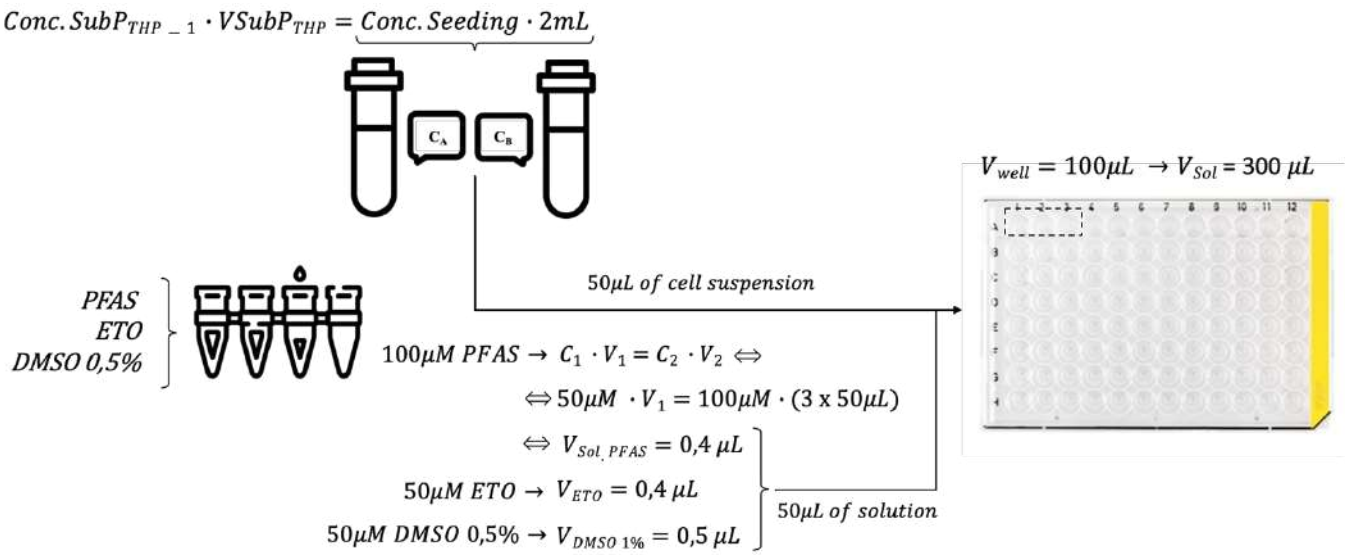


Figure 23. Ribbon (a) and surface (b) representation of PFAS in the structure of ERα. (From: DeWitt, J. C. (Ed.), 2015)

A3. Scheme of the test compounds and controls solutions for the MTS Assay.



A4. Population Doubling Time (PDT) Formula

$$PDT = \frac{T_{incubation} \times \ln(2)}{\ln\left(Conc_{Px+1} / Conc._{Px}\right)}$$

A5. Cell Viability (%) Formula

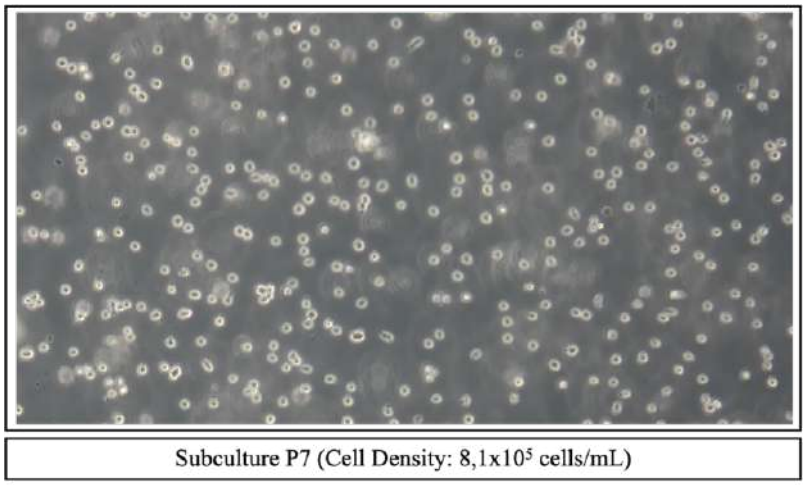
$$\overline{Abs\ Cells} <> x\ \%$$

$$\overline{Abs\ P_{THP\_1}PFAS} <> 100\%$$

$$Cell\ Viability(x) = \frac{\overline{Abs\ P\ THP{-}1\ PFAS} \times 100}{\overline{Abs\ Cells}}\ (\%)$$

A6. Microplate #2 (Non-Viable)

Miceoplate # 2 was seeded with $P_{THP\text{-}1}6$ (PDT=50h) which then underwent passage to create $P_{THP\text{-}1}7$. Later documented during cell culture maintenance.



The culture continued to progress normally despite the extremely low viability rate seen in this assay, suggesting that the abnormally high concentration of exposure is most likely what caused the cells' mortality.

Figure 24. Microscope Image of Subculture $P_{THP\text{-}1}7$.

Microplate #2

This cell culture was treated with triple the concentration of every test PFAS compound and control, which resulted in its complete unviability.

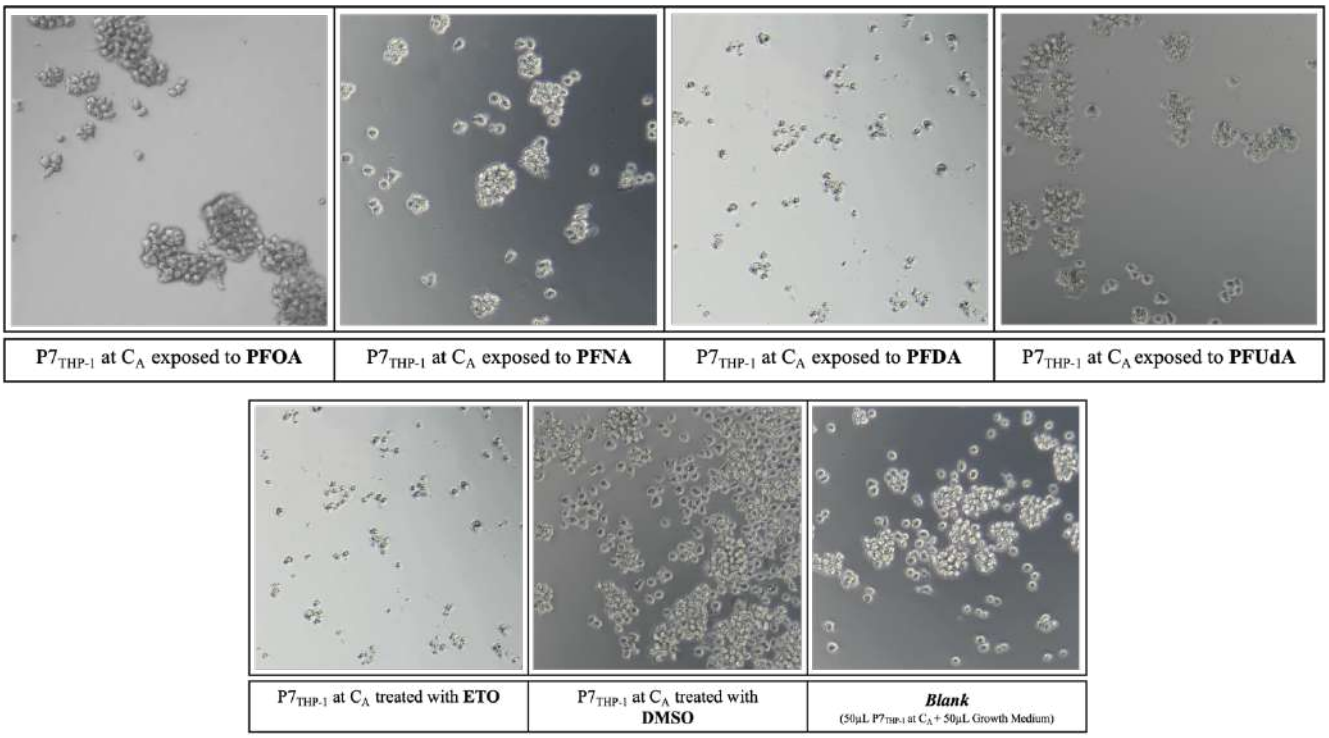


Figure 25. Microscope Image of Microplate #2 (seeded with $P_{THP\text{-}1}6$).