EFFECTS OF PERFLUORINATED COMPOUNDS ON THE HUMAN MAMMARY EPITHELIAL CELL LINE, MCF-10A, *IN VITRO*

EFFEKTER AV PERFLUORERTE FORBINDELSER PÅ DEN HUMANE BRYSTEPITELCELLELINJEN, MCF-10A, *IN VITRO*

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Abstract

There is a growing interest in assessing human health effects of persistent organic pollutants (POPs), and several studies have reported *in vitro* or *in vivo* effects related to cancer. The main aim of this study was to assess the potential effect of perfluorinated compounds (PFCs) on breast cancer development *in vitro*, using the MCF-10A human mammary epithelial cell line. Both MCF-10A monolayer and three-dimensional cultures were used to study the effect of PFCs in a time-dose-dependent manner. Flow cytometry was used to assess the cell cycle distribution of monolayer cultures. The percentage of cells in Sub-G_0/G_1, G_0/G_1, S and G_2/M were analyzed after 24, 48 and 72 hours in cells treated with 0, 100, 200, 300, 400 and 500 µM perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA). In this study only high concentrations of PFOS, PFNA and PFDA affected the cells, demonstrated by an increased percentage of cells in Sub-G_0/G_1.

To further investigate the effect of PFCs, laser scanning confocal microscopy was used to study acini polarization and lumen formation in MCF-10A three-dimensional cultures, treated with 0, 0.6, 6 and 60 µM of the same PFCs as the monolayer cultures. Alterations in polarization were determined at day 8 and 12, and lumen formation at day 3, 5, 7 and 10. MCF-10A cells organize into three-dimensional cultures, generating acini, when grown on a laminin-rich extracellular matrix. Acini formation begins with the clearance of the inner cells by apoptosis, to form lumen, followed by polarization of the outer cells. The *in vitro* observations showed that acini associated cell polarization and lumen clearance were compromised in three-dimensional cultures exposed to PFOS, PFNA, PFDA and PFUnA, even at the lowest exposure doses. In addition effects on lumen formation were observed due to PFOA exposure. Overall, this study demonstrated a difference in sensitivity between MCF-10A monolayer and three-dimensional cultures, and suggested a potential effect of PFCs on breast cancer development.

Sammendrag

Persistente organiske miljøgifter og deres effekt på human helse har de siste årene fått økt oppmerksomhet, og en rekke studier har rapportert *in vitro* eller *in vivo* eksponerings effekter, inkludert kreftutvikling. Hovedformålet ved denne studien var å evaluere den potensielle effekten av perfluorerte forbindelser (PFCs) med hensyn på brystkreft *in vitro,* ved bruk av MCF-10A humane brystepitelceller. Både MCF-10A monolag og tredimensjonale kulturer ble benyttet for å studere effekten av PFCs i en tid-dose-avhengig tilnærming. Flow cytometry ble benyttet for å evaluere cellesyklusdistribueringen i monolagkulturer. Andelen celler i Sub-G_0/G_1, G_0/G_1, S og G_2/M ble analyserte etter henholdsvis 24, 48 og 72 timer av celler behandlet med 0, 100, 200, 300, 400 and 500 µM perfluoroktankarboksylsyre sulfonsyre (PFOS), perfluoroktylsyre (PFOA), perfluorononononansyre (PFNA), og perfluorodekansyre (PFDA) og Perfluoroundekansyre (PFUnA). Av de fem stoffene testet, påvirket PFOS, PFNA og PFDA cellene, ved å øke antall celler i Sub-G_0/G_1.

For ytterligere å undersøke effekten av PFCs, ble det benyttet laser skanning konfokal mikroskopi for å studere polarisering og lumen dannelse i MCF-10A acini strukturer. De tredimensjonale kulturene ble behandlet med 0, 0,6, 6 og 60 µM av de samme stoffene brukt på monolagkulturer. Endringer i acini polarisering ble analysert ved dag 8 og 12, og lumen dannelse ved dag 3, 5, 7 og 10. MCF-10A celler danner tredimensjonale strukturer ved kontakt med laminin-rik ekstracellulær matrix, som gir opphav til acini strukturer. Acini dannelsen initieres ved apoptose av det indre laget med celler for dannelse av lumen, etterfulgt av polarisering av det ytre cellelaget. *In vitro* observasjonene indikerte komprimert polarisering og lumen dannelse i acini eksponert for PFOS, PFNA, PFDA og PFUnA, selv ved lave eksponerings konsentrasjoner. I tillegg ble det observert endringer i lumen dannelse ved PFOA eksponering. Resultatene presentert i denne studien viser med dette en forskjell i sensitivitet mellom MCF-10A monolag og tredimensjonale kulturer, samt en potensiell link mellom PFCs eksponering og økt risiko for brystkreft.

Contents

Abbreviations

µM:	Micromolar
µg:	Microgram
µl:	Microliter
Calcein AM:	Acetoxymethyl diacetylester of calcein
CLSM:	Confocal laser scanning microscopy
CO_2:	Carbon dioxide
BHb:	Bovine hemoglobin
BMb:	Bovine myoglobin
DAPI:	4'.6-diamidino-2-phenylindole
DCIS:	Ductal carcinoma *in situ*
DDT:	Dichlorodiphenyltrichloroethane
DMEM:	Dulbecco's Modifies Eagle Medium
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
DPBS:	Dulbecco's Phosphate-Buffered Saline
ECM:	Laminin-rich extracellular matrix
EGF:	Epidermal growth factor
EtBr:	Ethidium bromide
EtOH:	Ethanol
GM130:	Cis-Golgi matrix protein
H295R:	Human adrenocortical cell line
HepG2:	Liver hepatocellular cell line

MCF-10A:	Human mammary epithelial cell line
mg:	Milligram
miRNA:	Micro ribonucleic acid
ml:	Milliliter
mM:	Millimolar
mRNA:	Messenger ribonucleic acid
ng:	Nanogram
OCP:	Organochlorine pesticide
PCB:	Polychlorinated biphenyl
Pen/strep:	Penicillin/streptomycin
PFA:	Paraformaldehyde
PFC:	Perfluorinated compound
PFDA:	Perfluorodecanoic acid
PFNA:	Perfluorononanoic acid
PFOA:	Perfluorooctanoic acid
PFOS:	Perfluorooctanesulfonic acid
PFUnA:	Perfluoroundecanoic acid
POP:	Persistent organic pollutant
ROS:	Reactive oxygen species
TDLU:	Terminal ductal lobular units
Trition X-100:	Polyethylene glycol p-(1.1.3.3-tetramethylbutyl)-phenyl ether
Tween 20:	Polyoxyethylene (20) sorbitan monooleate
UN-ECE:	United Nations Economic Commission for Europe
β-Catenin:	Subunit of the cadherin protein complex

1. Introduction

1.1 Persistent organic pollutants

Persistent organic pollutants (POPs) is a collective term used to describe a wide range of chemicals, which during the last decades have got increased attention due to their potential hazardous effects on wildlife and humans. This has led to several international initiatives and in 1998 during a convention on Long-Range Transboundary Air Pollution, the United Nations Economic Commission for Europe (UN-ECE) developed a protocol that defines and addresses further measures to limit the release of POPs (Vallack et al., 1998). According to this protocol, POPs are defined as "…organic substances that: (i) possess toxic characteristics; (ii) are persistent; (iii) bioaccumulate; (iv) are prone to long-range transboundary atmospheric transport and deposition; and (v) are likely to cause significant adverse human health or environmental effects near to and distant from their sources" (UN-ECE, 2010).

The UN-ECE protocol led to an increased awareness regarding POPs, and in 2001 the Stockholm convention on persistent organic pollutants was adopted as an international initiative to limit or reduce the production and release of POPs (Lammel and Lohmann, 2012). Improvement of the control and regulation of POPs are an important subject, because although several POPs have been banned, they still exists in the environment due to long half-lives and persistency to degradation (Jones and de Voogt, 1999). Certain POPs are also still used in some developing countries, like organochlorine pesticides (OCPs) used for mosquito control and agriculture, which raises concerns about environmental exposure (El-Shahawi et al., 2010). POPs have been proven to travel long distances through atmospheric and ocean transport, and can therefore pose a risk not only for the country using it but also globally (Vallack et al., 1998).

The ability of POPs to move across long distances has led to a concern regarding their global distribution, and biomonitoring programs have been initiated (Wu et al., 2008). Increased knowledge about the presence of POPs in the environment and their toxicological effects, have led to a stronger focus on the impact of POPs on ecosystems, and several of these studies have reported potential harmful effects on humans and wildlife (Li et al., 2006).

1.1.1 POPs and its relevance to the environment and human health

One of the concerns regarding POPs is their ability to bioaccumulate in certain organism, and even biomagnify in top predators (Mackay and Fraser, 2000). Bioaccumulation has been associated with harmful effects including low reproduction rates, reduced survival and growth in offspring (El-Shahawi et al., 2010). Several studies have also reported a decline in marine populations related to POPs, like dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) (Wren, 1991, Nakamaru et al., 2002, Vasseur and Cossu-Leguille, 2006).

POPs are not only known to pose a risk to the environment and wildlife, but also to human health. Human exposure, and thereby accumulation of POPs, is primarily through our diet, mainly due to intake of meat and fish (Kelly et al., 2007). Several POPs have also been detected in human serum, blood, tissue and breast milk and in some cases at high concentrations (Hardell et al., 2010, Porta et al., 2008, Porta et al., 2012). The concentrations of POPs in humans will vary among different individuals, depending on lifestyle, but by being present over time they can potentially cause adverse health effects, such as changes in reproduction, development, hormone balance and even carcinogenic effects (Li et al., 2006).

1.1.2 POPs and cancer

Cancer is one of the major health burdens on a global perspective, and one of the leading causes of death worldwide (Siegel et al., 2012). Cancer is a complex disease, often caused by a combination of genetic and environmental factors. Studies have however shown that only 5-10 % of all types of cancer is solely genetically determined, emphasizing the effect of environmental factors, including exposure to POPs (Perera, 1997). POPs may not be directly genotoxic, but may nevertheless increase the probability for cancer development (Hou et al., 2012). The development of cancer can be promoted through alterations in the DNA sequences directly, due to mutations or changes in the genetic code. However, recent studies have shown that environmental toxins, like POPs, most likely promote cancer through interactions beyond the DNA sequence (Baccarelli and Bollati, 2009).

Certain POPs, such as DDT and its metabolites, have the ability to alter the DNA sequence by triggering over-production of reactive oxygen species (ROS), which can lead to oxidative DNA damage and thereby change the DNA sequence (EJ et al., 2013). Both *in vivo* and *in vitro* studies have also shown that DDT can initiate cell apoptosis by increasing the

production of ROS, promoting cancer development (Shi et al., 2010, Khan et al., 2000). POPs may also have the ability to change factors involved in gene regulation, and thereby change the gene expression without changing the genes itself (Mathur et al., 2002).

Factors that play an important role in gene expression like DNA methylation, histone modifications and miRNAs can all potentially be altered due to exposure to POPs. Remodeling of the DNA structure could lead to changes in gene expression due to the accessibility of transcription factors to the sequence (Hou et al., 2012). Studies performed by Rusiecki et al. (2008) have shown that there is a strong correlation between the concentration of POPs in human serum and the degree of global DNA hypermethylation. Demonstrating that POPs have the potential to affect factors involved in DNA regulation.

POPs can also increase the risk of cancer development by acting as a endocrine disruptor by for example mimicking estrogen, affecting breast development, and thereby increase the likelihood of breast cancer (Townson, 2004). Reports have indicated that *in utero* exposure can alter normal development, and therefore increase the risk for both breast- and testicular cancer in the offspring (Brody and Rudel, 2003, Hardell et al., 2006). Although there have been several studies on exposure to POPs and cancer, only a fraction of these chemicals have been tested, and new and emerging POPs are still being detected (Muir and Howard, 2007). This emphasizes the need to further evaluate and study new POPs, in order to identify their potential risk to the environment and human health.

1.2 Perfluorinated compounds

Perfluorinated compounds (PFCs) have recently been classified as a group of POPs, representing a potential risk to the environment and human health. PFCs are chemicals where the hydrogen atoms in the carbon backbone is replaced with fluorine, creating extremely stable fluorine-carbon bonds, making the chemicals resistant to biological degradation (Stahl et al., 2011). PFCs belong to a diverse group of chemicals that has been used in industrial applications for more than 50 years. They are extensively used in fabric, paper coating and paint, leading to the detection of several PFCs in the environment, bioaccumulating through food-chains and found at high concentrations in both animals and humans (Kannan, 2011). Several PFCs have been reported to be globally distributed, and certain compounds, like perfluorooctanesulfonic acid (PFOS), have been detected in a wide range of species, including birds and fish in a variety of ecosystems (Giesy and Kannan, 2001). PFCs have also been

measured in human blood, breast milk and tissue, confirming human exposure to PFCs, which give rise to concerns regarding adverse health effects (Fromme et al., 2009).

The exposure to PFCs starts already *in utero*, and studies have shown that exposure during pregnancy and in the suckling period leads to an increased body burden in the offspring, potentially causing harmful health effects (Fromme et al., 2010). Exposure early in life has been shown to cause adverse health impacts on children, showing that even low-level exposure both *in utero* and later in childhood can have a potential effect on cancer development, reproduction and the immune system (Damstra, 2002). Similar results have been reported in exposure studies with PFCs, including increased risk for breast cancer, changes in hormonal production and a potential immunotoxic effect (Corsini et al., 2011, Bonefeld-Jorgensen et al., 2011, Kraugerud et al., 2011).

1.2.1 The toxicology of perfluorinated compounds

How the different PFCs affect human health depends on their toxicological properties. Although most PFCs have similar structure, the length of the carbon backbone and the type of functional group attached differ among compounds. This gives each compound a unique physical, chemical and biological property (Buck et al., 2011). Some PFCs have been studied extensively; their global distribution, effects on human health, and concentration in tissue (Stahl et al., 2011). There are however still PFCs where very little information is available, particularly knowledge about their effects on human health. Further studies are therefore needed.

1.2.1.1 Perfluorooctanesulfonic acid

Perfluorooctanesulfonic acid (PFOS), consisting of 8 carbon atoms, connected to a sulfonic acid (Figure 1), is one of the most studied PFCs. It has a high surface activity, and is both hydro- and lipophobic due to the long alkyl chain, which makes it useful for industrial production of textiles, coating and food packaging (Wang et al., 2009). A large amount of PFOS have been produced, and it is therefore globally distributed in the environment (Giesy and Kannan, 2001). The global distribution of PFOS has also led human exposure, which gives concerns regarding the long term effect of exposure (Rumsby et al., 2009). Studies have shown that PFOS has a longer half-life compared to other PFCs with a different length of the

carbon backbone or another functional group (Olsen et al., 2007). The chemical properties of PFOS also allow it to interact with different amino acids in proteins, which have been shown to inhibit pathways like intracellular communications, through gap junctions (Hu et al., 2002). The specific length of its carbon-chain also enables binding through hydrophobic interaction with bases in the groove of DNA, potentially inhibiting transcription (Zhang et al., 2009).

1.2.1.2 Perfluorooctanoic acid

Perfluorooctanoic acid (PFOA) is another PFCs that has been extensively studied, but in contrast to PFOS, its toxicological properties have been associated with hormone disruption. PFOA consist of an 8 carbon-chain with a carboxylic acid attached (Figure 1). It has mainly been used in the manufacturing of Gore-Tex and Teflon due to its resistance to extreme temperatures and stresses (Steenland et al., 2010). Biomonitoring has showed that PFOA is present in human blood and wildlife, and toxicological studies have reported exposure effects on development and hormone regulation (Zhao et al., 2010). PFOA's toxicological properties have been reported to affect normal mammary gland development in CD-1 mice, leading to decreased development of the mammary epithelium (White et al., 2011). Alterations in the mammary gland development due to PFOA exposure have been linked to hormone production, including increased serum progesterone levels and elevated response to exogenous estradiol (Zhao et al., 2010).

1.2.1.3 Perfluorononanoic acid

Perfluorononanoic acid (PFNA) consists of a 9 carbon-chain with a carboxyl group at the end (Figure 1), making it similar but longer than PFOA. Although the two PFCs have similar structure, they have been known to possess different properties. *In vivo* studies of rats have shown that PFNA is eliminated slower than PFOA (Kudo et al., 2001). PFNA has primarily been used in the production of fluoropolymers, but it is also a potential degradation product of fluorotelomer alcohols, which makes PFNA widespread in the environment (Fang et al., 2009). A study performed on human HepG2 cells to determine the effect of PFCs on ROS production showed that only PFNA caused an increase in DNA damage at cytotoxic concentrations. In contrast to PFOS and PFOA, which did not affect ROS production (Eriksen et al., 2010). PFNA has also been linked to increased levels of apoptosis and oxidative stress in the spleen of rats (Fang et al., 2010).

1.2.1.4 Perfluorodecanoic acid

Perfluorodecanoic acid (PFDA) is composed of a 10 carbon-chain with an carboxyl group (Figure 1), and it has mainly been used for the production of commercial wetting agents and flame retardants due to its stability and tolerance to high temperatures (Nelson et al., 1992). *In vivo* studies of PFDA has shown that in possess more toxic properties and has a longer half-life when exposed to rats compared to shorter PFCs (Ohmori et al., 2003). PFDA has been found in both liver and blood, and it has been shown to enter blood cells and bind to amino acid residues of the main polypeptide chain in bovine hemoglobin (BHb) and myoglobin (BMb) (Qin et al., 2011). Binding of PFDA led to destruction of hydrogen bonds, causing a change in the secondary structure, which can lead to loss of normal physiological activity in BHb and BMb (Qin et al., 2011). PFDA has also been associated with mechanistic activation of lipid and redox signaling pathways, and mRNA down regulation in mouse livers (Maher et al., 2008).

1.2.1.5 Perfluoroundecanoic acid

Perfluoroundecanoic acid (PFUnA) consists of an 11 carbon-backbone, with a carboxyl group, making it one of the longer PFCs (Figure 1). PFUnA is mainly released to the environment as a breakdown product of grease-proof coatings used on food packaging and furniture (Buck et al., 2011, Olsen et al., 2011). There have been few studies of PFUnA, and little is known about its potential toxicological properties. It has been measured on a global scale, but often at lower concentrations compared to other PFCs (Butt et al., 2010, Gebbink et al., 2011). PFUnA has however been detected in human blood, which emphasizes the need to further study new and emerging POPs to establish their potential harmful properties (Olsen et al., 2012).

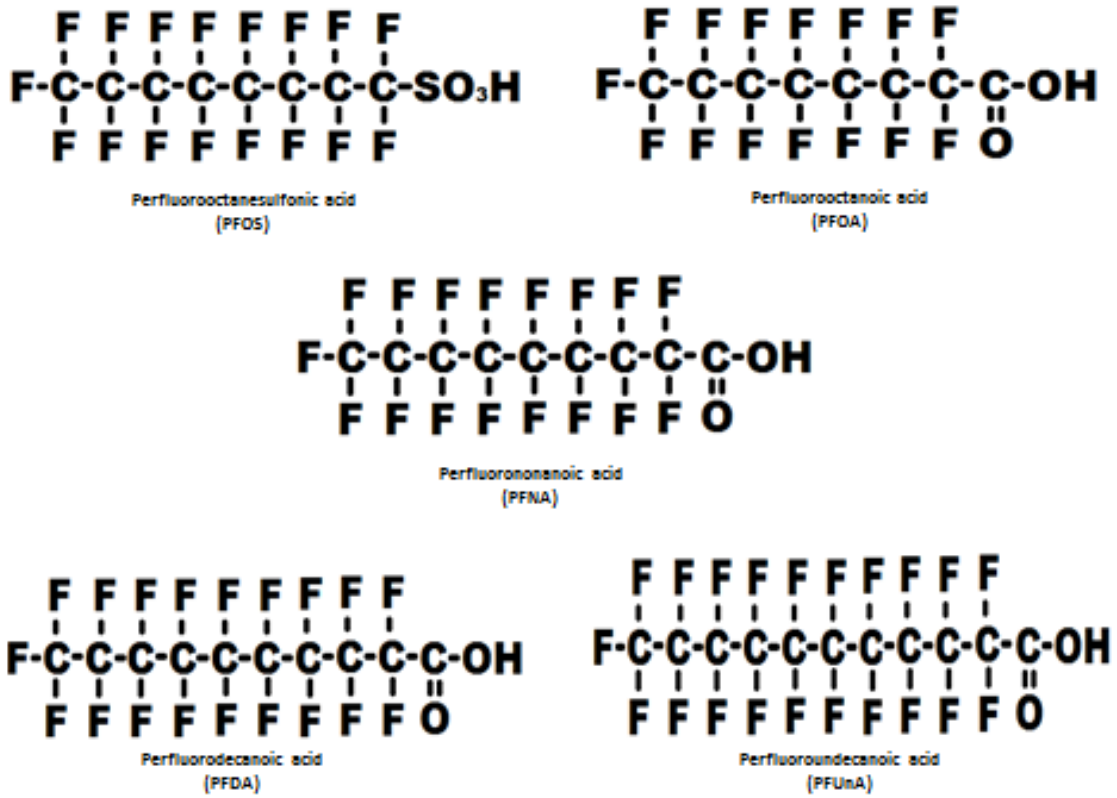

Figure 1: *Chemical structure of PFOS, PFOA, PFNA, PFDA and PFUnA.*

1.3 MCF-10A as an *in vitro* system to assess the toxicological effects of PFCs

Extensive biomonitoring of POPs have demonstrated the presence of PFCs in humans, and various studies have established a link between the presence of PFCs and hazardous health effects, including cancer development. However, little is known about how these compounds contribute to the morphological or genetic alterations seen in cancer *in vivo* (Stahl et al., 2011). To better understand the effect of PFCs on cancer development there is a need for further studies. The MCF-10A mammary epithelial cell model mimics many aspects of epithelial cells, which is the origin of most human cancers (Debnath and Brugge, 2005). MCF-10A is therefore a valuable model for toxicological studies assessing exposure effects on morphological or genetic alterations.

MCF-10A is a spontaneously immortalized human mammary epithelial cell line derived from the fibrocystic breast tissue of a 36-year-old female patient (Soule et al., 1990). MCF-10A cells have the ability to grow as monolayer when cultured in tissue culture plastic, and as three-dimensional cultures when in contact with laminin-rich extracellular matrix (ECM) (Figure 2). Three-dimensional cultures of MCF-10A replicates several aspects of epithelial organization, and primarily the glandular architecture *in vivo* (Debnath et al., 2003). The three-dimensional cultures give rise to acini-like spheroids that resembles terminal ductal lobular units (TDLU) *in vivo* (Figure 3), which is one of the main components in the human breast when fully developed (Inman and Bissell, 2010).

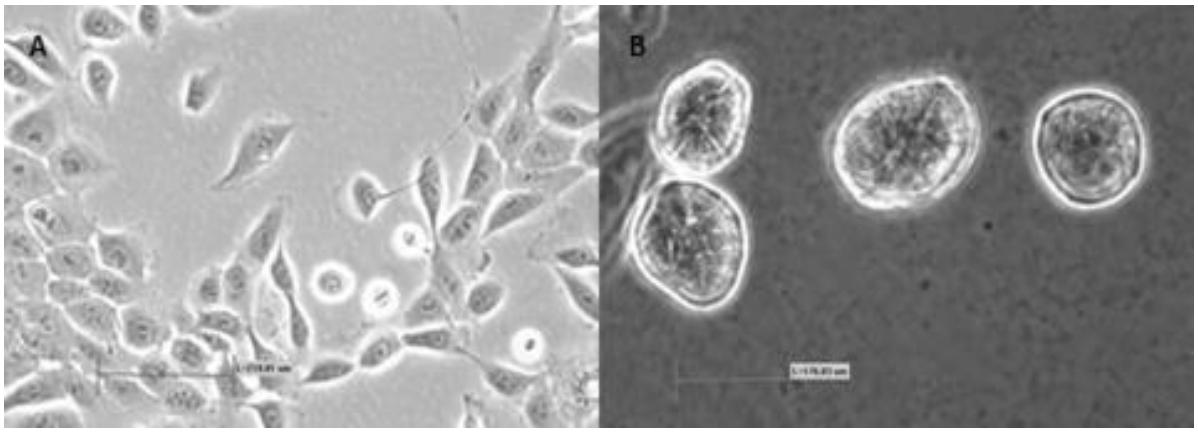

Figure 2: *Phase-contrast imaging of the morphology of MCF-10A cells grown as monolayer (A) and as three-dimensional cultures on ECM (B). Scale bars 70 µm.*

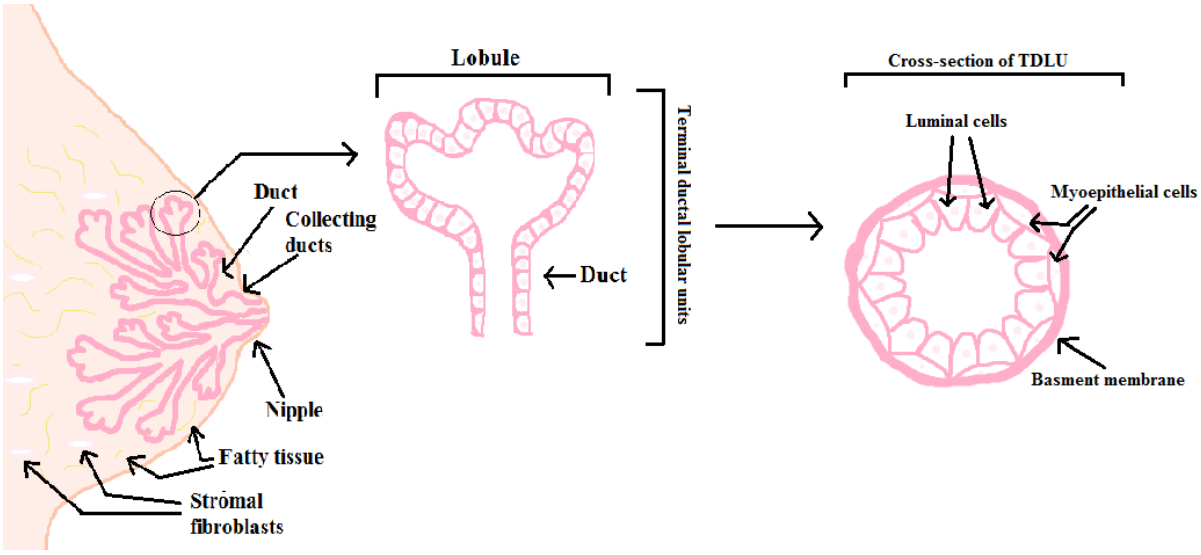

Figure 3: *Structure of the mammary gland, the terminal ductal lobular units and cross section of TDLU, showing the primary cells in normal ducts.*

When cultured on ECM, MCF-10A cells undergo several processes that include polarization, differentiation, lumen formation and growth arrest resulting in well-organized acini structures. Allowing insight to how extracellular factors interact with the development of epithelial tissue architecture (Imbalzano et al., 2009). By mimicking the structure of TDLU and undergoing several aspects vital for epithelial architecture, MCF-10A can also be used to mimic structural changes associated with breast cancer development, when induced to genetic alterations or hazardous chemicals (Shaw et al., 2004). Changes that include loss of polarization, filling of the luminal space and changes in structural organization, alterations considered as hallmarks for epithelial cancer (McCaffrey and Macara, 2011).

These changes are also vital for normal tissue development, in the breast especially, which are often altered due to changes in the micro-environment that surrounds the cells (Bissell and Hines, 2011). The micro-environment is known to be an active contributor to cancer development, and the breast tissue in particular is often more susceptible compared to other epithelial tissues, due to normal development and alterations during puberty, pregnancy and lactation. Extracellular factors in the breast can therefore to a greater extent contribute to malignancy (Ronnov-Jessen and Bissell, 2009). This makes three-dimensional cultures, like MCF-10A, valuable models for studying how extracellular factors, or compounds like PFCs, in the micro-environment can affect cancer development.

1.3.1 Modeling epithelial breast architecture and cancer in three-dimensional models

Throughout life the human tissue are replaced and repaired to maintain the organization and architecture of epithelial cells, and loss of epithelial structure is often associated with cancer development, which can be studied using three-dimensional models (Chanson et al., 2011). An important property of MCF-10A cells is their ability to undergo a polarized organization to form acini structures, important for normal tissue activity (Figure 4). Polarity is an essential part of the epithelium, which permits the cells to divide into distinctive apical and basolateral domains, allowing specific protein and signaling organization important for normal organ function (Liu et al., 2005). Disruption of polarity can therefore affect several aspects of normal cell or tissue activity by causing changes in pathways, including the ROS production, which can lead to genomic instability, increasing the risk for cancer development (Yamada and Cukierman, 2007).

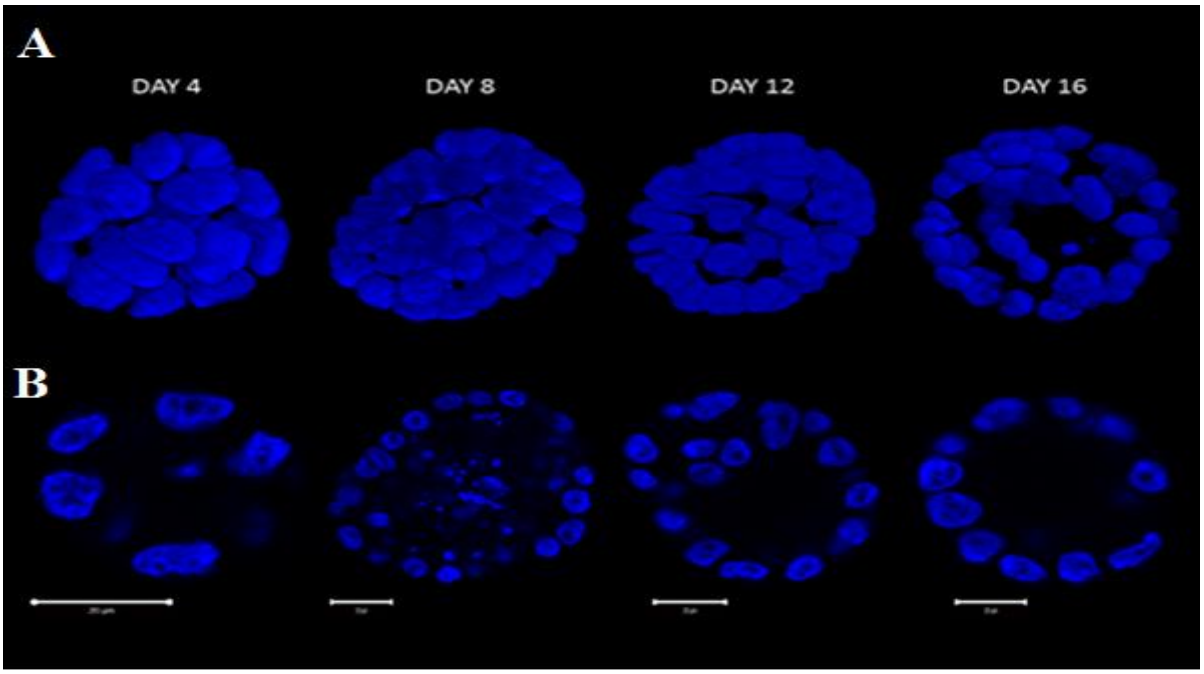

Figure 4: *Confocal microscopic imaging of MCF-10A acini (A) and confocal cross sections on MCF-10A acini (B) cultures on ECM from days 4-16; DAPI (blue). Illustrating cells proliferation (day 4) polarizing to form an outer layer of cells (day 8), followed by lumen formation through apoptosis (day 12) and formation of a mature acini structure (day16) Scale bars 20 µm*

Structural changes like lumen filling is also associated with increased cancer risk in epithelial acini structures (Debnath et al., 2002). Epithelial acini structures are defined by a spherical monolayer of cells that enclose a hollow lumen, which is formed due to selective apoptosis of cells located in the center of the structure (O'Brien et al., 2002). Apoptosis plays an important part of both normal gland development and breast cancer. Regulating the balance between proliferation and cell loss, and when disturbed can lead to filling of the lumen and malignancy (Kumar et al., 2000). Lumen filling plays and important part in breast cancer development, being an early sign of increased proliferation and inhibited apoptosis, which can lead to expression of oncogenes and increased risk for migration and invasiveness (Underwood et al., 2006).

Both filling of the lumen and lack of polarization are structural changes important in cancer development, which can be studied using the MCF-10A model (Debnath and Brugge, 2005). These structural changes can also occur due to changes in the micro-environment *in vivo*, which have been shown to influence several aspects of normal mammary gland development and breast cancer (Weigelt and Bissell, 2008). Structural changes that compounds like Benzo[a]pyrene has induced in MCF-10A (Siriwardhana and Wang, 2008), showing that exposure to POPs can potentially affect breast architecture.

1.4 Aims of study

Because of the extensive industrial use of PFCs and the persistency of these compounds, they are ubiquitous in the environment and a potential threat to human health. PFCs have also been demonstrated to be present in human breast milk and blood, and can thereby conceivably constitute a part of the micro-environment in the breast. PFCs can for this reason potentially affect the glandular architecture, increasing the risk of breast cancer.

The principle objective of this study was to investigate the effect of PFCs on breast tissue architecture *in vitro* with focus on alterations in polarization and lumen formation in three-dimensional cultures of MCF10-10A cells.

Secondary objectives were to compare effects of PFCs between MCF-10A monolayer and three-dimensional cultures. Additionally, this study aimed to contribute with new knowledge concerning exposure effects caused by specific PFCs (e.g. PFOS, PFOA, PFNA, PFDA and PFUnA), since these compounds are considered as new and emerging POPs.

To achieve these goals, three experiments with MCF-10A human mammary epithelial cells were preformed:

- Characterization of effects on cell cycle distribution in monolayer cultures
- Characterization of effects on polarization in three-dimensional cultures
- Characterization of effects on lumen formation in three-dimensional cultures

2. Materials and methods

The cell line used in this study was MCF-10A, a human mammary epithelial cell line (passage 29 to 34). It was a kind gift from Professor Finian Martin, University College Dublin (UCD). Handling of MCF-10A cells was done in accordance to protocols provided by Prof. Martin and Sara Ann McNally, UCD.

2.1 Chemicals

Heptadecafluorooctanedulfonic acid potassium salt (PFOS) (purity ≥ 98.0 %), perfluorooctanoic acid (PFOA) (purity > 96.0 %), perfluorononanoic acid (PFNA) (purity > 97.0 %), perfluorodecanoic acid (PFDA) (purity > 98.0 %) and perfluoroundecanoic acid (PFUnA) (purity > 95.0 %) were all purchased in powder from Sigma-Aldrich® (St Louis, MO, USA)

2.2 Preparation and exposure of monolayer cell cultures

2.2.1 Care and passage MCF-10A

Cells were maintained as a monolayer in 10-cm^2 tissue culture Falcon™ plastic dishes (BD Bioscience, Bedford, MA, USA) in growth medium (10 ml per dish): 250 ml Dulbecco's Modifies Eagle Medium (DMEM) (GIBCO®, Invitrogen, Paisley, UK), 250 ml Hans F-12 (GIBCO®, Invitrogen), 5 % horse serum (GIBCO®, Invitrogen), 20 ng/ml epidermal growth factor (EGF) (PeproTech, Rocky Hill, NJ, USA, stock solution 100 µg/ml in sterile water), 0.5 µg/ml hydrocortisone (Sigma- Aldrich®, stock solution 1 mg/ml in EtOH), 100 ng/ml cholera toxin (Sigma-Aldrich®, stock solution 1 mg/ml in sterile water), 10 µg/ml insulin (Sigma Aldrich®, stock solution 10 mg/ml in sterile water containing 1 % glacial acetic acid), and 5 ml pen/strep (GIBCO®, Invitrogen). All additives and mediums were mixed and filtered through a 0.2 µm filter (Millipore, Billerica, MA, USA) to sterilize.

For splitting and seeding of the cells the growth medium was aspirated and the cells washed with 10 ml of sterile Ca^{2+}, Mg^{2+} -free Dulbecco's Phosphate-Buffered Saline (DPBS) (GIBCO®, Invitrogen). The DPBS was aspirated and 2 ml of 1X trypsin solution

(0.05 % Trypsin, GIBCO®, Invitrogen) was added and the cells incubated in a 5 % CO_2 humidified incubator at 37ºC for 10-25 minutes. The extent of the trypsinization was checked every 3-5 minutes using a light microscope (Nikon TMS microscope, phase contrast, Melville, NY, USA) and gently tapping the dish. Once cells were dislodged the trypsin was transferred to a 15 ml Falcon™ tube (BD bioscience), and the plate rinsed with 3 ml of resuspension medium (250 ml DMEM, 250 ml Hans F-12, 20 % horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml Insulin, and 5 ml Pen/Strep). The cells were spun down at 800 rpm for 3 minutes in a Beckman GS-6R centrifuge (Beckman coulter, Ltd., Brea, CA, USA). The medium was aspirated and the cells were resuspended carefully in 5-6 ml growth medium. 1 ml of the growth medium with cells was added per 10-cm^2 dish containing 9 ml of growth medium. The cells were incubated in a 5 % CO_2 humidified incubator at 37ºC, a 1:5 passage ratio became confluent after 48 hours, and a 1:6 passage ratio after 72 hours.

2.2.2 *In vitro* exposure of MCF-10A

The test compounds were diluted into 600 mM in dimethyl sulfoxide (DMSO) (Sigma-Aldrich®), divided into 100 µl aliquots, and stored at -20ºC until use. Before the exposure, MCF-10A cells were trypsinized, as previously described, and resuspended in 3 ml of resuspension medium. The cells were counted using a haemocytometer (Bürker counting chamber, Glaswarenfabrik Karl Hecht GmbH & Co KG - "Assistent", Sondheim / Rhön, Germany) and added to a 50 ml Falcon™ tube (BD bioscience), containing growth medium, at a final concentration of 10 000 cells/ml. 3 ml of this mixture was plated in each well in a 6-well Falcon™ plate (BD bioscience) and the cells were allowed to attach for 24 hours in a 5 % CO_2 humidified incubator at 37ºC.

After 24 hours the medium in each well was replaced with fresh growth medium and the stock solutions of the compounds were diluted in DMSO and growth medium to yield the final concentration of 500, 400, 300, 200, 100 and 0 µM for each compound. The final exposure concentrations were decided based on pilot exposure studies of MCF-10A cells. The cells were exposed in triplicate wells to the compounds final concentration, control cells were exposed to 0 µM, containing only 0.1 % DMSO. The cells were incubated in a 5 % CO_2 humidified incubator at 37ºC for 24, 48 and 72 hours. At the end of each incubation-time cells were harvested for flow cytometry. The experiment was repeated three times.

2.3 Flow cytometry

MCF-10A cells treated with the test compounds for 24, 48 and 72 hours, as described above, were harvested for cell cycle phase distribution assayed by Flow cytometry. Upon harvesting, the supernatant for each exposure concentration was collected in a separate centrifuge tube (VWR International, Radnor, PA, USA), and the cells washed with 1 ml DPBS. The DPBS was aspired and added to the collected supernatant, then 500 µl trypsin was added to each well and the cells incubated in in a 5 % CO_2 humidified incubator at 37ºC for 1-5 minutes. The cells were checked every minute with gently tapping the plates. Once the cells were dislodged, the trypsin was added to the centrifugation tube, and the wells rinsed with 500 µl resuspension medium, which was mixed with the supernatant.

The tubes were then centrifuged at 300 xg for 10 minutes in a Heraeus Multifuge X1R (Thermo Fisher Scientific, Hudson, NH, USA) and the supernatant discarded. The cells were resuspended by gently tapping on the tube, to dissolve the cell pellet, followed by an additional resuspension in 3 ml DPBS and centrifugation at 300 xg for 10 minutes. The process was then repeated an additional time. Pelleted cells were resuspended in the remaining liquid and fixed by drop-wise adding 1 ml 70 % ice-cold ethanol (Kemetyl, Halden, Norway) (in dest. water) while wortexing. After all the cells were fixed, another ml of ice-cold 70 % ethanol was added. The Cells were then kept on ice for 1 hour, before storage at 4ºC for at least 24 hours before analysis.

On the day of analysis the cells were kept on ice, 3 ml DPBS was added to the tube and it was centrifuged at 300 xg for 10 minutes. The cells were then resuspended in residual ethanol, followed by 1-3 hour incubation in the dark at room temperature with 1 ml of propidium iodide solution (50 ng/ml RNAse (Qiagen, Crawley, UK) and 50 µg/ml propidium iodide (Sigma- Aldrich®) in DPBS). The proportion of cells in each cell cycle phase was determined by propidium iodide florescence using the Coulter® EPICS XL-MCL™ flow cytometer (Beckman Coulter), evaluating 10, 000 events per sample. Forward and light scatter data were collected in a linear mode. Fluorescence data was collected in the FL3 channel on a linear scale. Doublets were excluded using gating. Side- and forward-light scatter parameters were used to identify the cell events.

2.4 Preparation and exposure of three-dimensional cultures

2.4.1 Three-dimensional growth of MCF-10A cells

In order to generate three-dimensional cultures, 8-well Lab-Tek® glass chamber slides (Thermo Scientific) were pre-coated with growth factor reduced matrigel™ (BD Biosciences). The matrigel™ was thawed on a 1:1 mixture of ice and water for 3-4 hours prior to coating. The 8-well glass camber slides were then coated with 45 µl matrigel™ per well while kept on ice, followed by incubation in a 5 % CO_2 humidified incubator at 37ºC for 50-60 minutes to solidify. Monolayer cultures of MCF-10A cells were trypsinized, as previously described, and resuspended in 1 ml assay medium (250 ml DMEM, 250 ml Hans F-12, 2 % horse serum, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 5 ml pen/strep). The cells were counted using a haemocytometer and added to a stock of assay medium supplemented with 2 % matrigel™ and 5 ng/ml EGF at a final concentration of 10 000-15 000 cells/ml. 400 µl of this mixture was seeded on each well on the 8-well glass chamber slides coated with matrigel™ (4000-6000 cells/well).

The cells were cultured in a 5 % CO_2 in a humidified incubator at 37ºC and the medium changed every 4. day with assay medium supplemented with 2 % matrigel™ and 5 ng/ml EGF. The day the cells were seeded correspond to day 0. The medium was changed on day 4, 8, 12, 16 etc. The cells started to form spheres by day 3-6, hollow lumen by day 4-10, and complete acini structures were formed by day 10-14.

2.4.2 *In vitro* exposure of MCF-10A

The test compounds were diluted into 600 mM in DMSO, divided into 100 µl aliquots, and stored at -20ºC until use. Before the exposure, the stock solutions were diluted in DMSO and growth medium to yield the final concentration of 60, 6, 0.6 and 0 µM for each compound. The final exposure concentrations were decided based on pilot exposure studies of MCF-10A cells. For each exposure and the controls two separate matrigel™ populations were used, the control cells were exposed to 0 µM, containing only 0.1 % DMSO. The cells were incubated in a 5 % CO_2 humidified incubator at 37ºC for up to 12 days and re-exposed during medium change every 4. day. Cells were fixated at day 8 and 12 for antibody staining, and used for live staining at day 3, 5, 7 and 10 assessed by confocal laser scanning microscopy (CLSM).

2.5 Confocal laser scanning microscopy

2.5.1 Immunofluorescence staining

Cells were fixed by using 3-4 % paraformaldehyde (PFA) (Sigma- Aldrich®). The medium was aspirated from each well of the chamber slide and 200 µl PFA was added, and the cells incubated for 15-20 minutes at room temperature. The PFA was then removed, and the cells washed twice with 500 µl DPBS. The DPBS was aspirated and 300 µl DPBS-0.5 % Triton X-100 (250 µl Trition X-100 (Sigma- Aldrich®) in 50 ml DPBS) was added to each well for 5-10 minutes on a shaker at room temperature. The DBPS-0.5 % Triton X-100 was removed, and the cells washed twice with 500 µl DPBS. The cells were then incubated with 500 µl blocking solution (500 µl normal goat serum (Molecular Probes®, Invitrogen) in 9.5 ml DBPS) for 1 hour on a shaker at room temperature. The excess blocking solution was removed and 100 µl primary antibody, (purified mouse anti-β-Catenin or purified mouse anti-GM130 (BD Bioscience)), diluted in blocking solution (1:50) was added and the cells incubated for 2 hours on a shaker at room temperature.

After incubation the primary antibody was aspirated, and the wells washed twice with 300 µl DBPS-0.1 % tween® 20 (50 µl tween® 20 (Sigma- Aldrich®) in 50 ml DBPS) for 3-5 minutes on a shaker. The DBPS-0.1 % tween was then removed, and the slides incubated with 100 µl primary antibody (Alexa-Fluor 555 goat anti-mouse IgG or Fluorescein goat anti-mouse IgG (Molecular Probes®, Invitrogen)) diluted in blocking solution (1:750 or 1:1000) in the dark on a shaker for 40 minutes at room temperature. The secondary antibody was then removed, and the wells washed twice with 300 µl DPBS-0.1 % tween for 3-5 minutes on a shaker, followed by counterstaining with DAPI (Molecular Probes®, Invitrogen) for 30 seconds. The DAPI was aspirated, and one drop of Slow Fade® Gold antifade reagent (Molecular Probes®, Invitrogen) was added to each well. The cells were stored for up to 3 days in the dark at 4ºC before analysis. To characterize alterations in polarization based on morphology in exposed MCF-10A acini, the distribution patterns of the marker protein GM130 and β-catenin, associated with mammary epithelial polarization, were investigated using CLSM.

2.5.2 Vital dyes staining

For live staining analysis with vital dyes the medium was aspirated and each well washed with 500 µl DBPS. The cells were then incubated for 10-15 minutes with 50 µl Ethidium Bromide (EtBr) (1 µM EtBR (Sigma- Aldrich®) in DBPS) and 200µl acetoxymethyl diacetylester of calcein (Calcein AM) (10 nM Calcein AM (Molecular Probes®, Invitrogen) in DPBS) in the dark at room temperature. The EtBr-Calcein AM solution was then aspirated, and the cells washed twice with 500 µl DBPS, and 200 µl DBPS was added to the wells to avoid desiccation. To characterize alterations in lumen formation based on morphology in exposed MCF-10A acini, the balance between vital and apoptotic cells in the luminal space, determined by Calcein AM and EtBr staining, were investigated immediately after staining using CLSM.

2.5.3 Analysis of exposed three-dimensional cultures

MCF-10A cells treated with test compounds and stained with antibodies toward either β-catenin or GM130, as described above, were harvested for analysis of polarization in acini structures. Acini structures stained with vital dyes, as described above, were used for analysis of lumen formation. In all assays, a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) was used to assess and visualize the acini structures and staining; images were acquired using LSM software ZEN (Carl Zeiss) version 2009. Fluorescence from DAPI was excited at 405 nm by a Diode laser, fluorescence from EtBr and Alexa 555 at 514 by an Agron laser and Calcein AM and Fluorescein at 488 by an Agron laser. On the day of analysis multiple overview CLSM pictures were taken of each separate matrigel™ population for each exposure concentration and controls. For β-Catenin, GM130 and vital staining at least 80 acini structures were visualized per separate matrigel™ population.

There were also taken several pictures of individual acini structures for each exposure concentration and controls. The number of non-polarized acini, stained with β-catenin or GM130, was determined by scoring acini structures with aberrant immunofluorescence patterns in the overview pictures. The number of acini with aberrant lumen formation was determined by scoring multiple transformed acini structures, with no or few apoptotic cells in the lumen and an irregular shape in the overview pictures. In all cases, prior to the scoring, all pictures were coded, randomized. The pictures were then scored blind.

2.6 Statistical analysis

Statistical analysis was performed using JMP 9 software (SAS Institute Inc, Cary, NC, USA). When necessary, data were log-transformed to achieve a satisfactory fit to the normal distribution (Shapiro Wilk test). Log-transformation of the percentage of affected cells or acini exposed to PFOS, PFOA, PFNA, PFDA and PFUnA assessed by flow cytometry and vital dye, gave a better fit to the normal distribution and was used in the statistical assessment. Otherwise the percentage of acini assessed by GM130 and β-catenin gave a satisfactory fit to the normal distribution.

The exposure effect on cell cycle distribution and acini structures was evaluated using general linear models (GLMs). Log-transformed percentages of affected cells/acini or percentage of affected cells/acini were dependent variables. Independent categorical variables were time points analyzed and exposure compounds. Log-transformed exposure dose and the interaction between log-transformed exposure dose and exposure compound were continuous variables. In addition, within each exposure compound the effect of exposure dose on mean percentage of altered acini structures was evaluated by Welch's t test in cases of unequal variance (Levene's test). In cases of equal variance Dunett's test were used. Dose-response relationships were assessed for each exposure compound by simple linear regression analysis. P-values <0.05 were considered statistically significant.

3. Results

3.1 Cell cycle distribution

Flow cytometric analysis revealed that exposure of MCF-10A monolayer cultures for 24, 48 and 72 hours to PFOS, PFNA and PFDA resulted in a significant increase of cell populations in the sub-G_0/G_1 fraction (Figure 5-7), and a decrease in the number of cells in the G_0/G_1, S and G_2/M phases (Figure 8). Increased cell populations in the sub-G_0/G_1 fraction were observed after exposure to concentrations higher than 300 µM (PFOS), 400 µM (PFNA) and 200 µM (PFDA), respectively (Figure 5-7). No significant effect were observed after PFOA and PFUnA exposure.

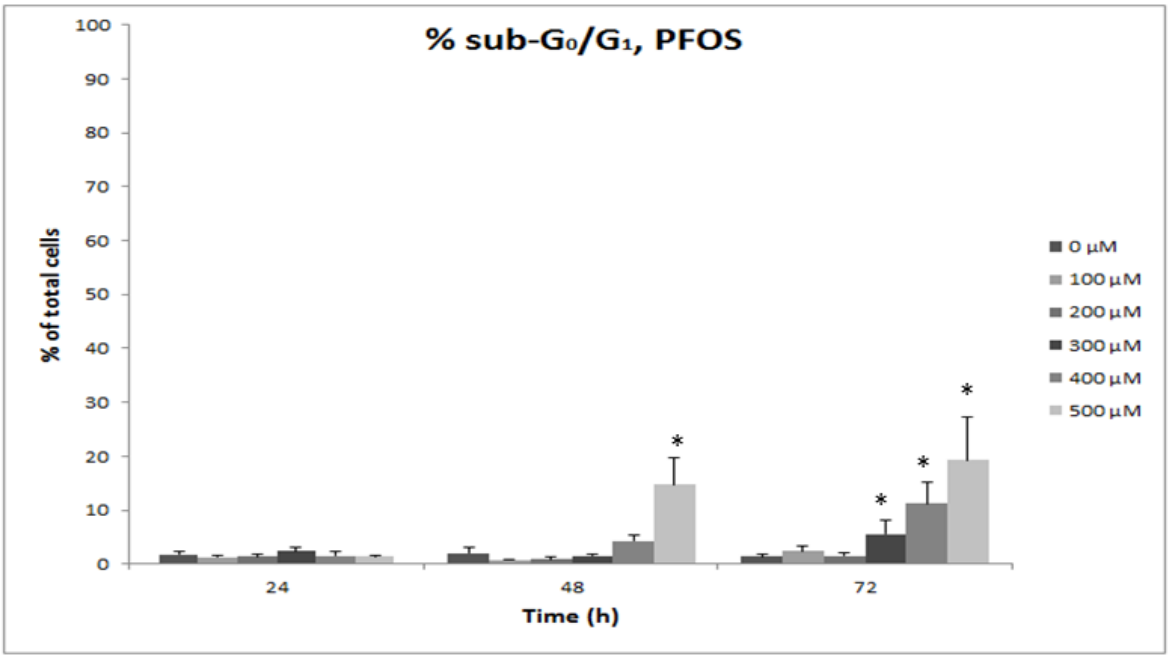

Figure 5: *Mean percentage ±SE of MCF-10A cells in the sub-G_0/G_1 fraction after 24, 48 and 72 hours exposure to six concentrations of PFOS. Each experiment was performed three times (n=3).* Significantly different from control (Welch's t test; $p < 0.05$).*

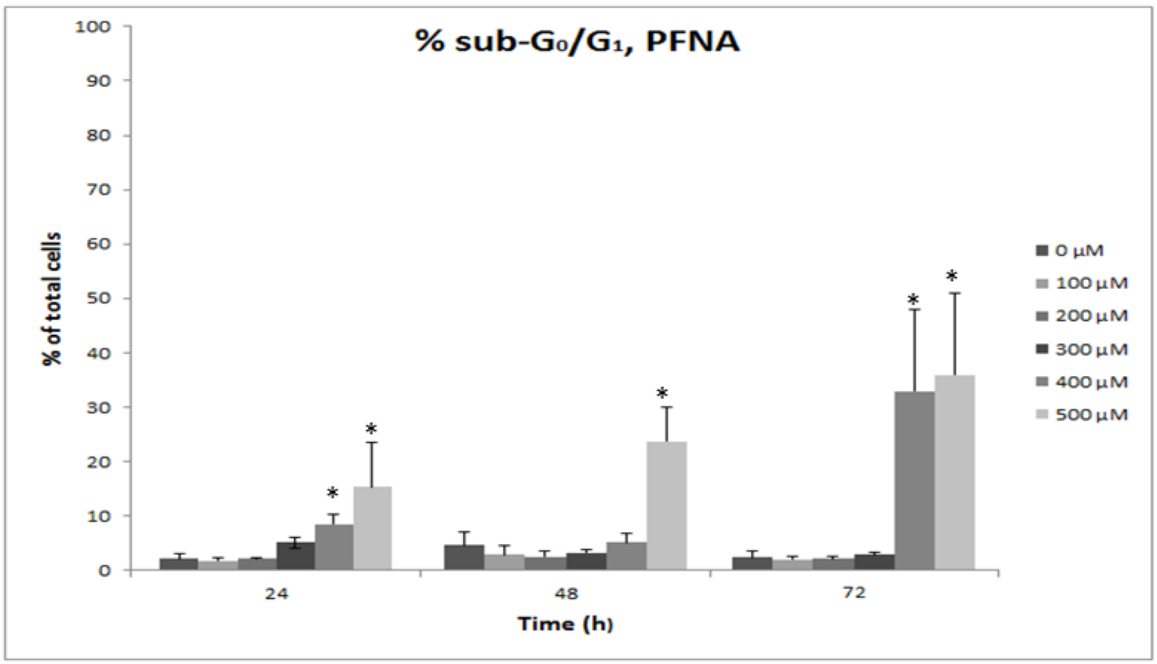

Figure 6: *Mean percentage ±SE of MCF-10A cells in the sub-G_0/G_1 fraction after 24, 48 and 72 hours exposure to six concentrations of PFNA. Each experiment was performed three times (n=3).* Significantly different from control (Welch's t test; $p < 0.05$).*

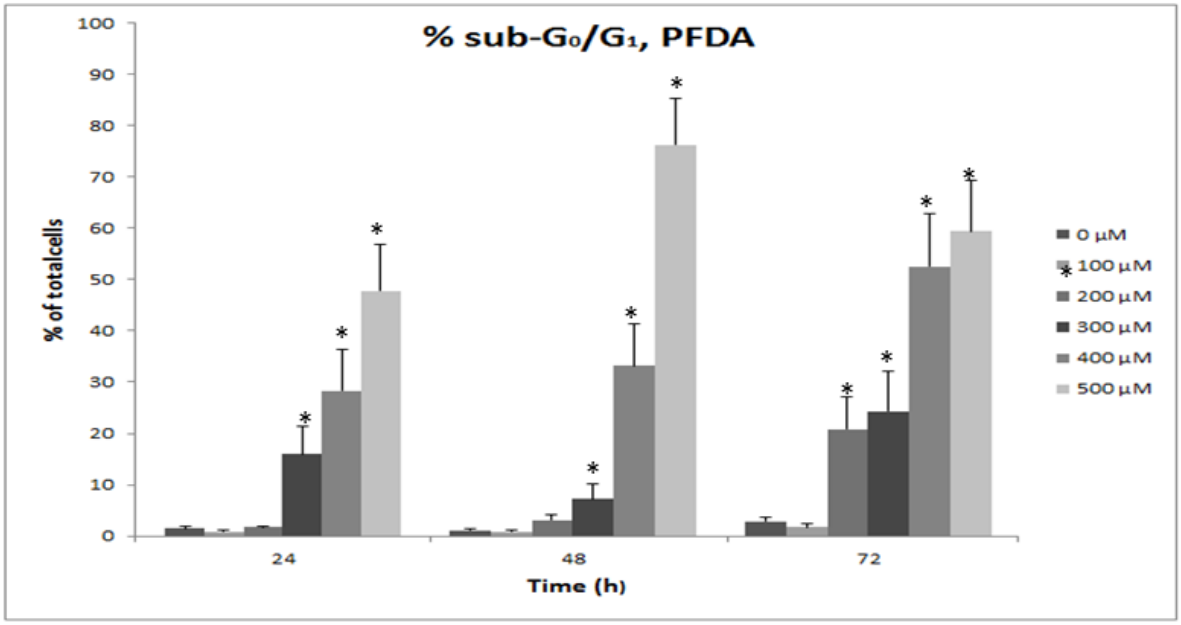

Figure 7: *Mean percentage ±SE of MCF-10A cells in the sub-G_0/G_1 fraction after 24, 48 and 72 hours exposure to six concentrations of PFDA. Each experiment was performed three times (n=3).* Significantly different from control (Welch's t test; $p < 0.05$).*

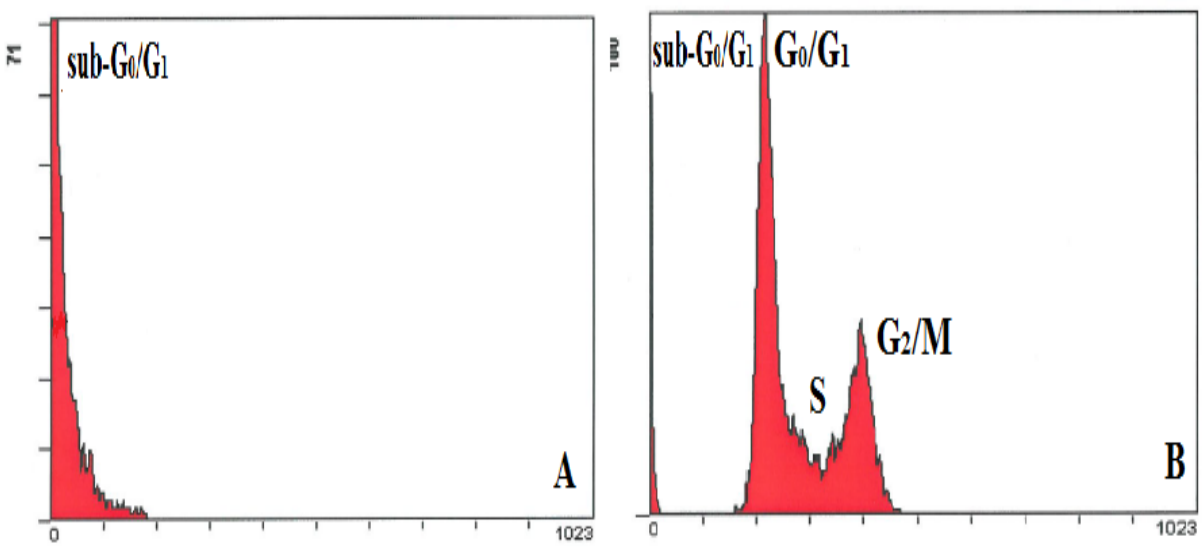

Figure 8: *Representative cell cycle distribution of MCF-10A monolayer cells exposed to perfluorinated compounds (500 µM) or their solvent controls (0 µM) for 48 hours. Cells were stained with PI and analyzed using flow cytometry. Few viable cells could be detected in the cells exposed to PFDA (A), whereas a normal cell cycle distribution was seen in the solvent control (B).*

3.2 Acini morphology

3.2.1 GM130 analysis

Based on the marker protein GM130, the data suggested that apical polarization in MCF-10A acini was significantly affected by exposure compound in three dimensional cultures (Table 1). The percentage of non-polarized acini structures increased with increasing log-transformed exposure doses and was significantly increased on day 12 day compared with day 8. In addition, the relationship between log dose and response significantly differed between compounds (Table 1).

The mean percentage of non-polarized acini structures were considerably higher in MCF-10A cultures exposed to 0.6 and 6 µM PFOS, PFNA and PFDA at both day 8 and 12 compared to the non-exposed cultures (Figure 9). The 6 and 60 µM exposure to PFUnA also lead to a small, but significant increase in the mean percentage of non-polarized acini structures at both time-points analyzed (Figure 9). The 60 µM doses of PFOS, PFNA and PFDA led to cell death, and the percentage of non-polarized acini structures at 60 µM could therefore not be quantified.

Table 1: *Parameter estimates with standard errors (SE), Sums of squares (SS) and degrees of freedom (df) for GLM for the impact of exposure, time and log-transformed dose on the percentage of non-polarized acini in MCF-10A three-dimensional cultures assessed by GM130 staining.*

Response	Model		R^2-Model	df	Parameter estimates	SE	SS	p-value
% Non-polarized acini structures	Intercept		0.95		21.05	0.294		<.0001
	Exposure	PFDA		4	8.712	0.787	6352	<.0001
		PFNA			8.037	0.787		<.0001
		PFOS			6.967	0.787		<.0001
		PFUnA			-11.75	0.707		<.0001
		PFOA			Ref	Ref		Ref
	Time	12		1	2.082	0.366	294.8	<.0001
		8			Ref	Ref		Ref
	Log Dose			1	2.827	0.119	5125	<.0001
	Log Dose × Exposure	PFDA		4	1.976	0.257	3060	<.0001
		PFNA			1.637	0.257		<.0001
		PFOS			1.411	0.257		<.0001
		PFUnA			-2.417	0.206		<.0001
		PFOA			Ref	Ref		Ref

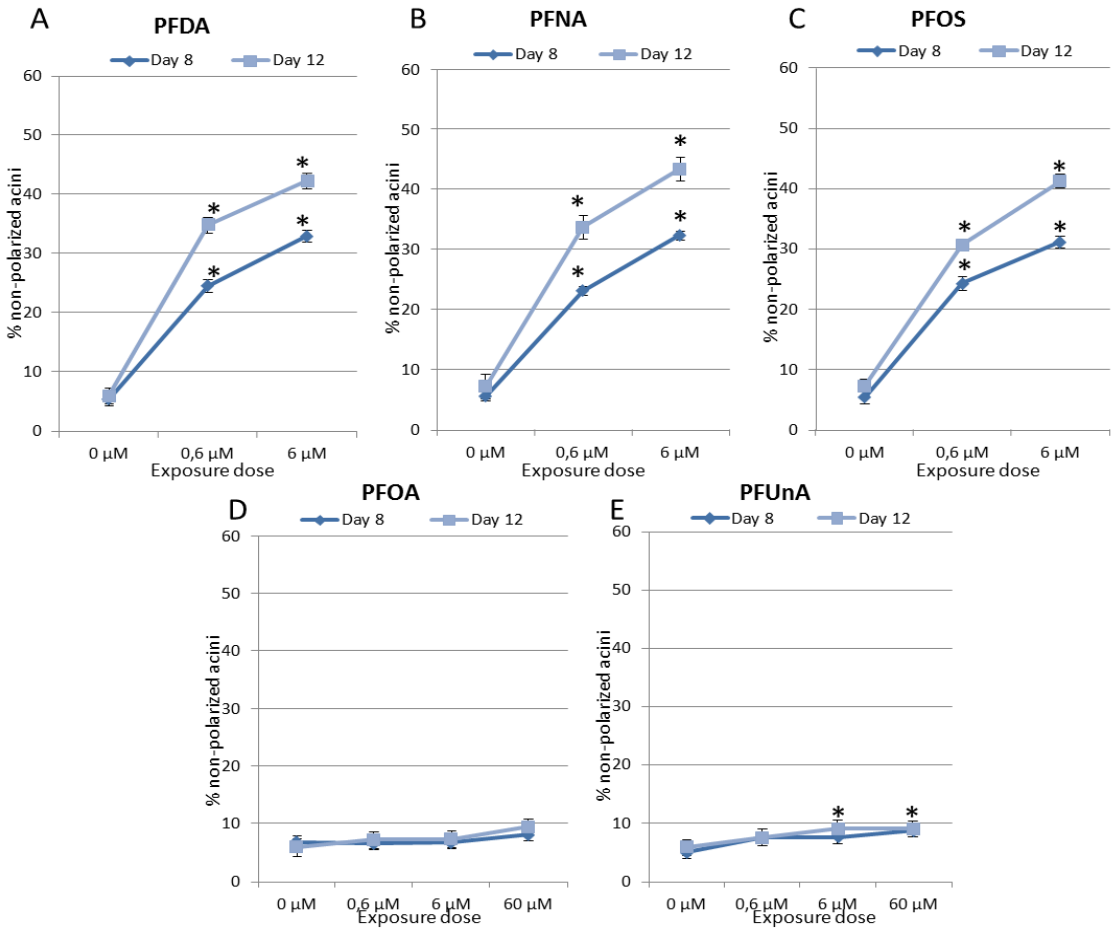

Figure 9: *Mean percentage ± SE of non-polarized acini exposed to 0, 0.6, 6 and 60 µM PFDA (A), PFNA (B), PFOS (C), PFOA (D) and PFUnA (E) for 8 and 12 days assessed by GM130 staining.* Significantly different from control (Welch's t test; $p < 0.05$).*

In solvent control MCF-10A three-dimensional cultures, GM130 was localized to the apical surface of the entire acini structure (Figure 10A). In three-dimensional cultures exposed to PFOS, PFNA, PFDA or PFUnA this uniform localization was disrupted and GM130 was randomly distributed in the acini structures (Figure 10B). The mean percentage of acini structures with aberrant distribution of GM130 was shown to significantly increase in MCF-10A three-dimensional cultures with increasing doses of PFOS ($p=0.0003$), PFNA ($p=0.0009$), PFDA ($p=0.0009$) and PFUnA ($p=0.04$) analyzed by Welch's t test. In addition, PFOS, PFNA, PFDA and PFUnA displayed a significant dose-response relationship assessed by simple linear regression analysis. Increasing doses of PFOA did not significantly affect acini structures by Welch's t test or simple linear regression analysis.

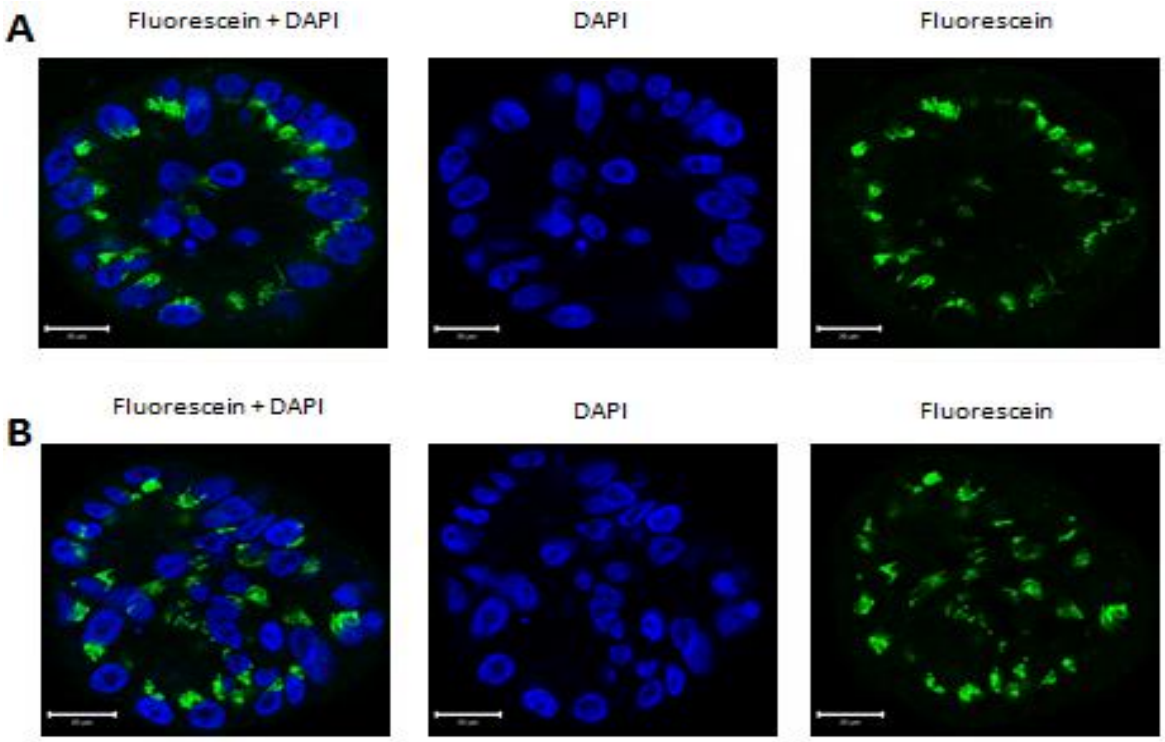

Figure 10: *Confocal microscopic imaging of MCF-10A acini stained for GM130 (green) and DAPI (blue). At day 8 and 12 GM130 localizes apically to Golgi apparatus in ECM-associated cells, reflecting polarized acini, demonstrated by the solvent control at day 12 (A). When exposed to PFOS, PFNA, PFDA or PFUnA GM130, although punctuate, is randomly distributed, demonstrated by acini exposed to 6 µM PFDA at day 12 (B). Scale bars 20 µm.*

3.2.2 β-catenin analysis

Based on the marker protein β-catenin, the data suggested that basolateral polarization in MCF-10A acini was significantly affected by exposure compound in three dimensional cultures (Table 2). The percentage of non-polarized acini structures increased with increasing log-transformed exposure dose and was significantly increased on day 12 day compared with day 8. In addition, the relationship between log dose and response significantly differed between compounds (Table 2).

The mean percentages of non-polarized cells were considerably higher in MCF-10A acini exposed to 0.6 and 6 µM PFOS, PFNA and PFDA at both day 8 and 12 than in the unexposed cultures (Figure 11). However, there were no apparent differences in the mean percentage of non-polarized cells between 0.6 and 6 µM in PFDA exposed cells on day 8 (Figure 11). The 60 µM doses of PFOS, PFNA and PFDA led to cell death, and the number of non-polarized cells at this concentration could not be quantified. Exposure with PFOA and PFOA and PFUnA did not affect basolateral polarization.

Table 2: *Parameter estimates with standard errors (SE), Sums of squares (SS) and degrees of freedom (df) for GLMs for the impact of exposure, time and log-transformed dose on the percentage of transformed acini in MCF-10A three-dimensional cultures assessed by β-catenin staining.*

Response	Model		R^2-Model	df	Parameter estimates	SE	SS	p-value
% Non-polarized acini structures	Intercept		0.92		21.80	0.479		<.0001
	Exposure	PFDA		4	11.13	0.957	6647	<.0001
		PFNA			6.568	0.957		<.0001
		PFOS			6,257	0.957		<.0001
		PFUnA			-11.37	0.859		<.0001
		PFOA			Ref	Ref		Ref
	Time	12		1	2.004	0.445	273.2	<.0001
		8			Ref	Ref		Ref
	Log Dose			1	2.826	0.145	5121	<.0001
	Log Dose ×	PFDA		4	2.259	0.313	3213	<.0001
	Exposure	PFNA			1.501	0.313		<.0001
		PFOS			1.366	0.313		<.0001
		PFUnA			-2.522	0.250		<.0001
		PFOA			Ref	Ref		Ref

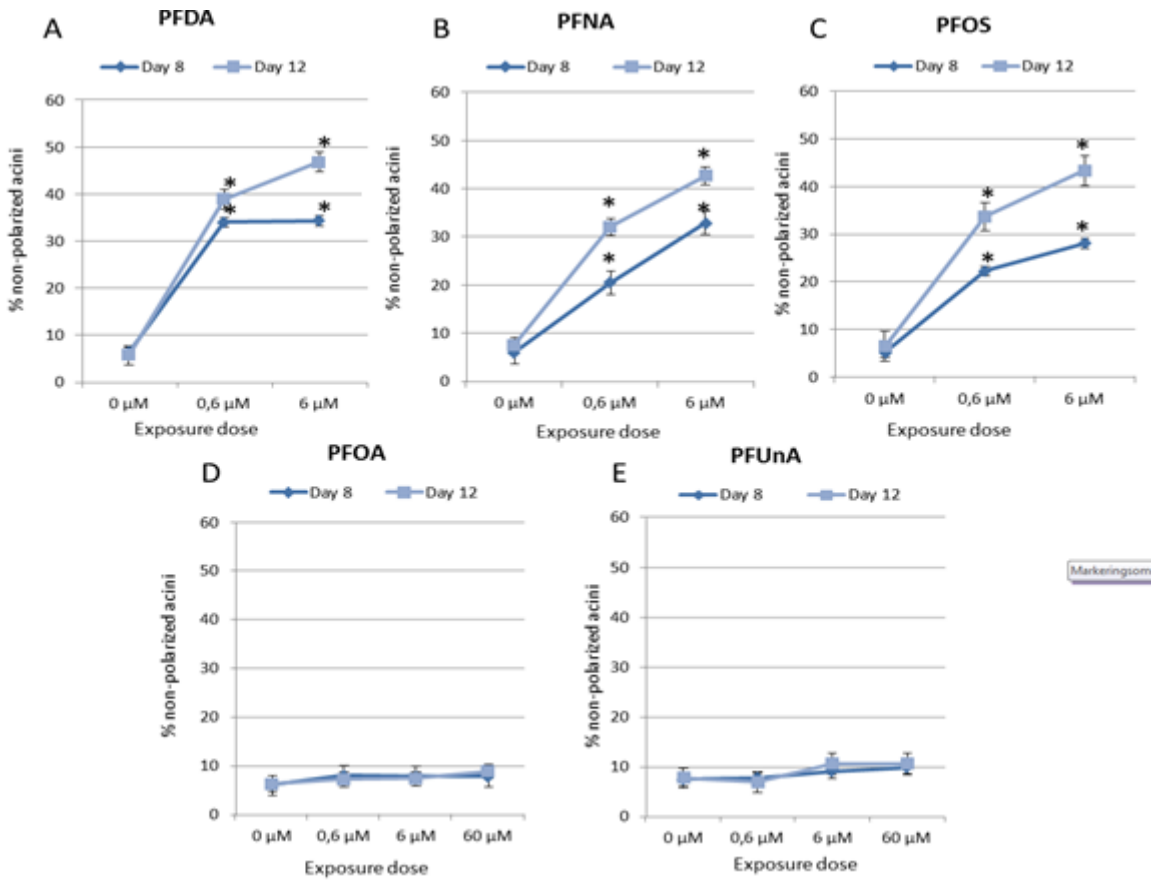

Figure 11:*Mean percentage ± SE of non-polarized acini exposed to 0, 0.6, 6 and 60 µM PFDA (A), PFNA (B), PFOS (C), PFOA (D) and PFUnA (E) for 8 and 12 days assessed by β-catenin staining.* Significantly different from control (Welch's t test; p < 0.05).*

In solvent control MCF-10A three-dimensional cultures, β-catenin was localized to the basolateral surface in acini structures, faced away from the luminal space (Figure 12A). In MCF-10A acini exposed to PFOS, PFNA or PFDA the polarization was disrupted and β-catenin was randomly distributed in the acini structures (Figure 12B). The mean percentage of non-polarized acini structures was shown by Welch's t test to significantly increase with increasing doses of PFOS (p=0.003), PFNA (p=0.008) and PFDA (p=0.0001). In addition the compounds were shown to affect polarization in a dose-dependent manner by simple linear regression analysis. No significant effect was observed after PFOA and PFUnA exposure.

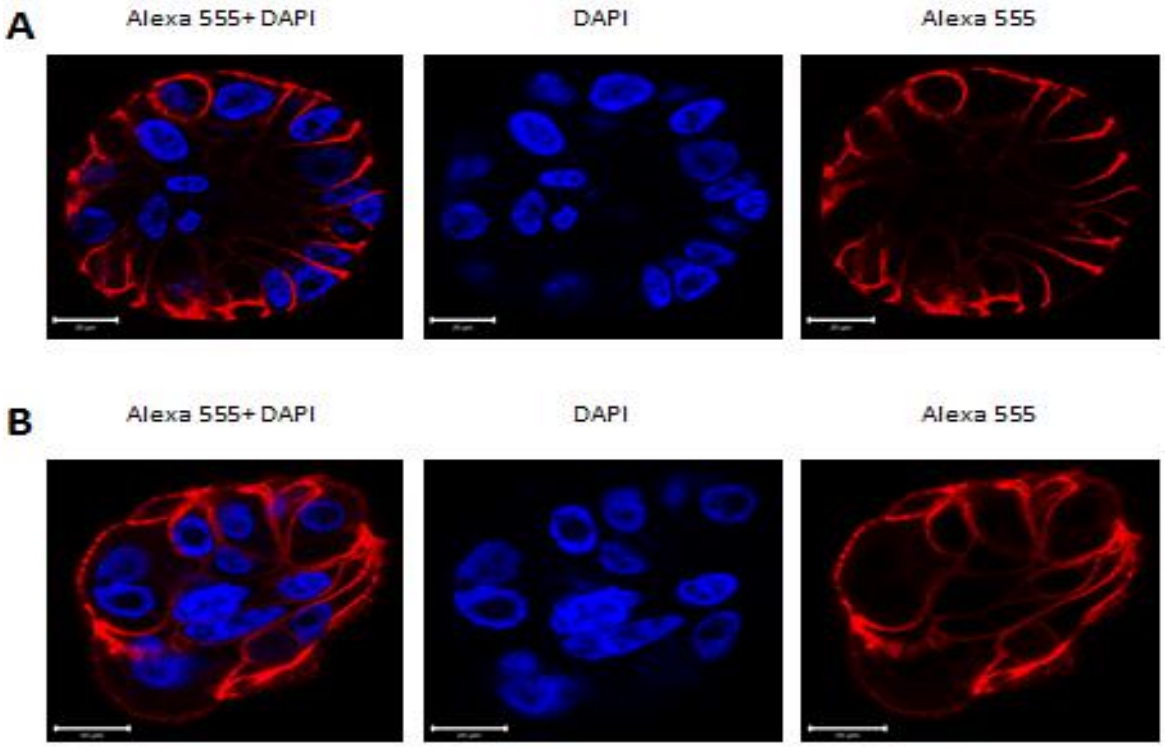

Figure 12: *Confocal microscopic imaging of MCF-10A acini stained for β-catenin (red) and DAPI (blue). At day 8 and 12 β-catenin redistributes to the basolateral membrane, demonstrated by the solvent control acini at day 12 (A), however when MCF-10A acini structures were exposed to PFOS, PFNA or PFDA, β-catenin was randomly distributed in the acini structure, demonstrated by acini exposed to 6 µM PFDA at day 12 (B). Scale bars 20 µm.*

3.2.3 Lumen formation

Based on vital dye staining, the data suggested that lumen formation in MCF-10A acini was significantly affected by time of exposure and exposure compound in three dimensional cultures (Table 3). The percentage of non-polarized acini structures increased with increasing log-transformed exposure dose and over time. In addition, the relationship between log dose and response significantly differed between compounds (Table 3).

Separate analyses of each exposure compound indicated that there was a clear effect on the mean percentages of transformed cells due to exposure to PFOS, PFNA and PFDA, and a marginal effect of PFOA and PFUnA (Figure 13). The 60 µM doses of PFOS, PFNA and PFDA led to cell death by day 7, and the number of non-polarized acini structures at this concentration could therefore not be quantified at day 7 and 10.

Table 3: *Parameter estimates with standard errors (SE), Sums of squares (SS) and degrees of freedom (df) for GLMs for the impact of exposure, time and log-transformed dose on the percentage of transformed acini in MCF-10A three-dimensional cultures assessed by vital dyes staining.*

Response	Model		R^2-Model	df	Parameter estimates	SE	SS	p-value
% transformed acini structures	Intercept		0.85		2.496	0.02		<.0001
	Exposure	PFDA		4	0.552	0.051	25.9	<.0001
		PFNA			0.158	0.052		0.0027
		PFOS			0.262	0.052		<.0001
		PFUnA			-0.463	0.048		<.0001
		PFOA			Ref	Ref		Ref
	Time	10		3	0.403	0.044	16.9	<.0001
		5			-0.528	0.043		0.2913
		3			-0.045	0.042		<.0001
		7			Ref	Ref		Ref
	Log Dose			1	0.145	0.007	34.5	<.0001
	Log Dose × Exposure	PFDA		4	0.090	0.015	10.5	<.0001
		PFNA			0.055	0.015		0.0007
		PFOS			0.042	0.015		0.0063
		PFUnA			-0.094	0.014		<,0001
		PFOA			Ref	Ref		Ref

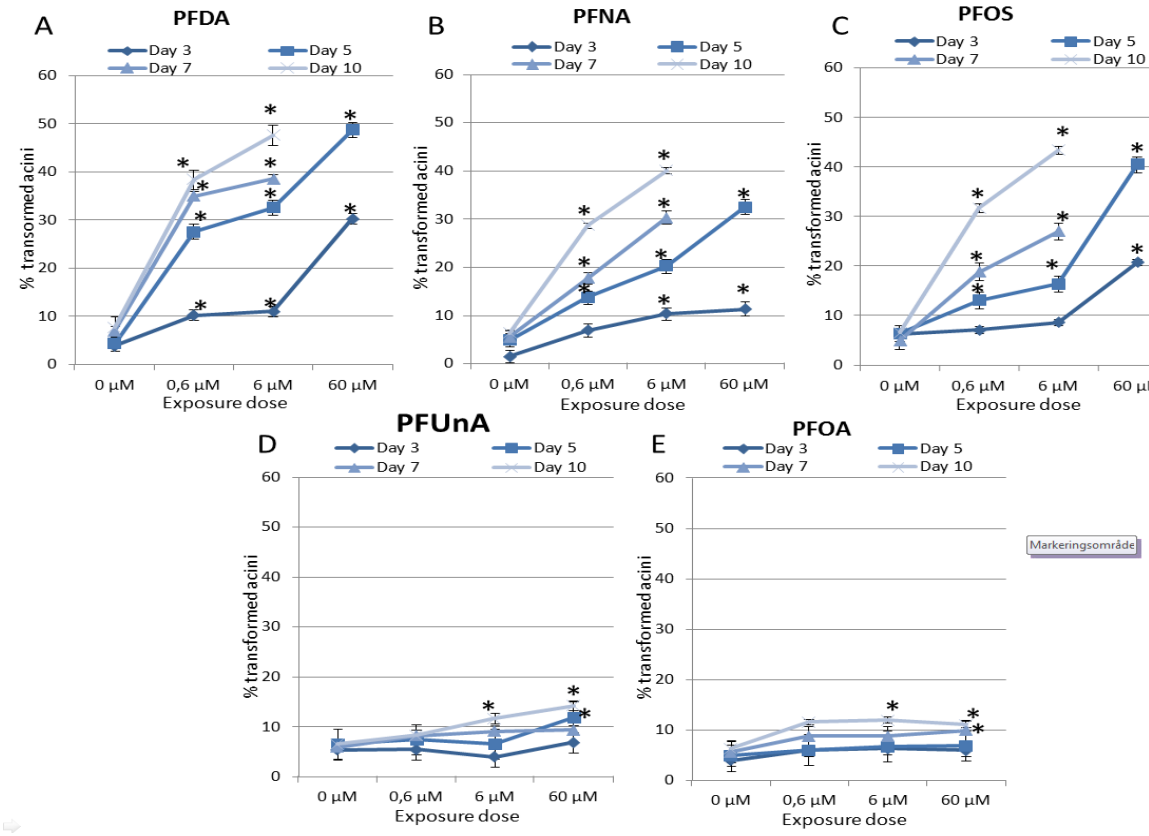

Figure 13: *Mean percentage ± SEM of transformed acini exposed to 0, 0.6, 6 and 60 µM PFDA (A), PFNA (B), PFOS (C), PFOA (D) and PFUnA (E) for 3, 5, 7 and 10 days assessed by vital dyes staining.* Significantly different from control (Welch's t test; $p < 0.05$).*

In solvent control normal formation of lumen occurred in MCF-10A acini (Figure 14A). In acini exposed to PFOS, PFOA, PFNA, PFDA or PFUnA the lumen formation was compromised, leading to low numbers of apoptotic cells in the luminal space (Figure 14B). The percentage of acini with compromised lumen formation was shown to significantly increase with increasing doses of PFOS ($p=0.0001$), PFNA ($p=0.0006$), PFOA (0.02), PFDA ($p=0.0001$) and PFUnA ($p=0.03$) by Welch's t test. In addition, all five compounds were shown to affect lumen formation in a dose-dependent manner by simple linear regression analysis. However, Dunett's test revealed that only the highest concentration of PFOA and PFUnA affected lumen formation, in contrast to PFOS, PFNA and PFDA where even the lowest dose affected lumen formation ($p<0.05$).

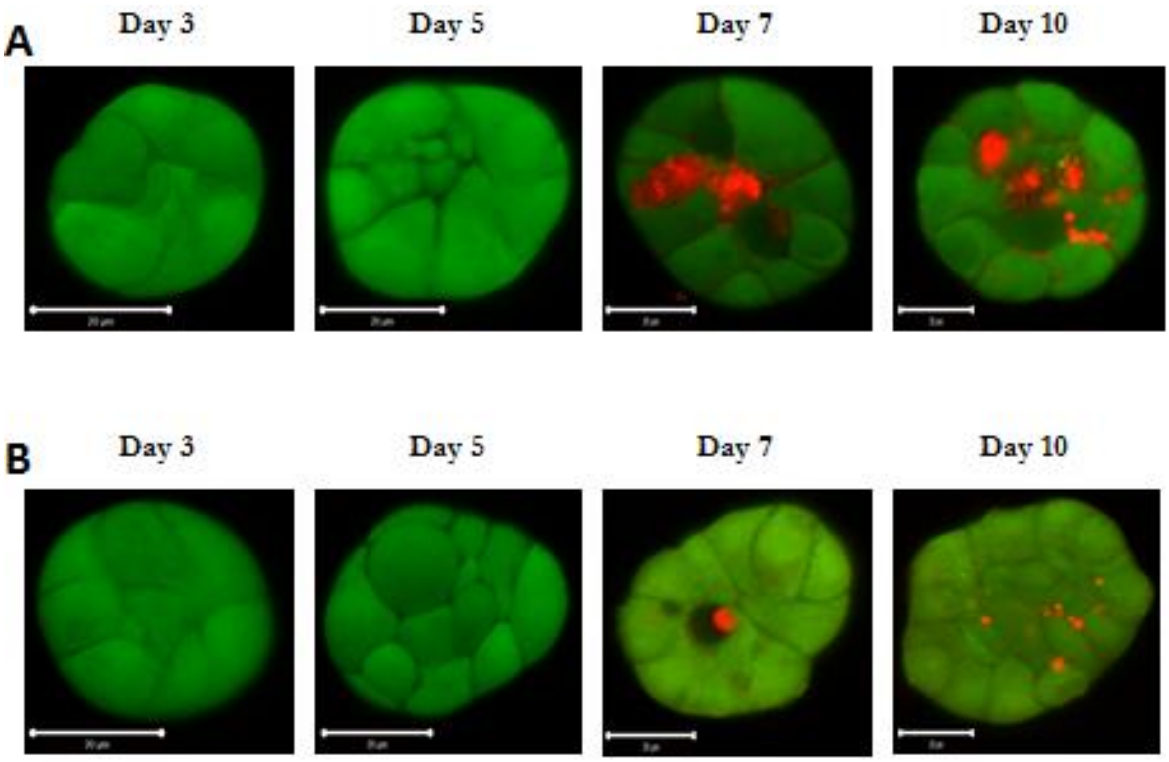

Figure 14: *Confocal microscopic imaging of MCF-10A acini stained with EtBr (orange) and Calcein AM (green). During lumen formation there is a distinctive formation of outer and luminal cell populations followed by apoptosis of luminal cells (red), demonstrated by solvent control acini (A). In acini exposed to PFOS, PFNA, PFDA, PFOA and PFUnA the cell populations were randomly distributed within the acini structure and low or no level of apoptosis was observed, demonstrated by acini exposed to 6 µM PFDA (B). Scale bars 20 µm.*

4. Discussion

The present study describes, for the first time, exposure effects of the compounds, PFOS, PFOA, PFNA, PFDA and PFUnA, on MCF-10A human mammary epithelial cells *in vitro.* Effects related to cell morphogenesis were found in both monolayer and three-dimensional cultures, but more pronounced in acini cultures. PFOS, PFNA and PFDA were found to affect all endpoints studied in both types of MCF-10A cultures. PFOA affected only lumen formation in three-dimensional cultures and PFUnA both lumen formation and to some degree polarization.

4.1 Cell cycle distribution

Cell division is usually strictly controlled by the cell cycle, to ensure correct cell and DNA replication, however, a defining characteristic of cancer cells is their ability to divide independently of the cell cycle (Clurman and Roberts, 1995). As such, it is not surprising that alterations in the cell cycle are common during malignancy (Park and Lee, 2003). The present study evaluated the effect of perfluorinated compounds on the cell cycle distribution in MCF-10A monolayer cultures. Three of the test compounds, PFOS, PFNA and PFDA, altered the number of cells in the sub-G_0/G_1 population, whereas PFUnA and PFOA had no significant effect on the cell cycle distribution. The flow cytometry results indicated that PFOS, PFNA and PFDA had a limited cytotoxic effect at high doses which was largely due to apoptosis.

An increase in apoptosis by these compounds has previously been demonstrated in both rats, mice and H295R cells, linked to triggering of oxidative stress and activation of apoptotic pathways (Feng et al., 2009, Dong et al., 2012, Kraugerud et al., 2011). Although the present results indicated an effect of PFCs on MCF-10A cell cycle distribution, studies have shown that several signaling pathways in monolayer cultures are fundamentally different from what is found in three-dimensional models and *in vivo* (Bissell et al., 2005). Monolayer cultures may therefore not fully reflect the exposure effect of PFCs *in vivo* (Weaver et al., 1996). However, two-dimensional cultures, like MCF-10A monolayer, can be useful to reveal potential exposure effects, and are valuable in combination with three-dimensional models to fully understand several aspects of cell response.

4.2 Acini morphology

Alterations in the morphology of the breast are important characteristics of cell response during cancer development and used as a diagnostic tool to identify malignancy (Schnitt, 2010). Malignancy of the breast is known to originate from lobular units, which during early stages of cancer development has been reported to display morphological alterations, including loss of polarity and compromised lumen formation (Cichon et al., 2010). Alterations that have been replicated using MCF-10A three-dimensional cell cultures, enabling studies of cancer development in the breast at *in vivo*-like conditions (Shaw et al., 2004). The present study reports that exposure to PFCs compromises acini morphology, including filling of the luminal space and reduced apical and basolateral polarity. Alterations in morphology suggest that exposure to PFCs impairs normal acini formation and organization of mammary epithelial cells, supported by studies of vital dye staining and the aberrant localization of the marker proteins GM130 and β-catenin.

These marker proteins also hold prognostic information regarding early stages of breast cancer (Lin et al., 2000, Nakamura, 2010). During normal tissue development, β-catenin constitutes a part of the basolateral junctions, located to the outer layer of cells, forming a distinctive basal and lateral surface away from the lumen (Nishioka et al., 2009). However, in MCF-10A three-dimensional cultures exposed to PFOS, PFNA, PFDA and PFUnA, β-catenin were relocated in the acini structures, an alteration in localization observed during breast cancer development (Geyer et al., 2010). Translocation of β-catenin from the baolateral surface throughout the structure has been reported to activate target genes associated with increased growth, invasion and cellular transformation (He et al., 1998, Lin et al., 2000).

Alterations in polarization and localization were also observed at GM130 staining of MCF-10A acini. GM130 is a cytoplasmic protein tightly bound to the Golgi apparatus, involved in correct formation of cell polarization (Nakamura, 2010). Normally the Golgi apparatus, thus GM130, is strictly localized to the apical cell side, faced inward to the luminal space (Friesland et al., 2013). However, in MCF-10A cultures exposed to PFOS, PFNA and PFDA, GM130 were present throughout the acini structures, which has been reported to occur during the initiation of cancer cell invasion (Chang et al., 2012). Taking the aberrant localization of both β-catenin and GM130 into consideration, the results indicate that exposure to PFCs lead to reduced polarization and alterations in acini morphology associated with early stages of breast cancer, known as ductal carcinoma *in situ* (DCIS) (Leonard and Swain, 2004).

DCIS in the breast has been defined as malignant epithelial cells restricted to the ductal system without any invasion to the surrounding environment (Quinn et al., 1997). The transition from normal epithelial cells to malignant cells found in DCIS has been reported to include increased viability (Burstein et al., 2004). Vital dye staining of acini structures was therefore used in addition to the marker proteins to further evaluate the effect of exposure on morphological alterations linked to breast cancer. The vital dye staining consisted of EtBr, which forms strong interactions with the DNA of apoptotic cells (Baskic et al., 2006), and Calcein AM, that rapidly enters viable cells and thereby get hydrolyzed into a intracellular fluorescence (Grieshaber et al., 2010). Vital dye enables therefore the evaluation of the relationship between viability and apoptosis. During normal acini formation, viable cells are strictly located around the luminal space, followed by apoptosis of the remaining cells to form lumen (Underwood et al., 2006). However, in three-dimensional cultures exposed to PFOS, PFOA, PFNA, PFDA and PFUnA, the number of apoptotic cells decreased leading to filling of the luminal space. A morphological alteration, which in combination with aberrant localization of the marker proteins GM130 and β-catenin, are long-established phenotypes of breast cancer (Debnath and Brugge, 2005).

Polarization and lumen formation in MCF-10A mammary acini are ongoing processes, leading to the formation of fully developed acini structures by day 16 (Debnath et al., 2003). Analysis of cell polarization on day 8 and 12, and lumen formation at day 3, 5, 7 and 10 represents therefore both early initiation and later organization of acini structures (Whyte et al., 2010). In the present study, all compounds displayed the ability to affect lumen formation. PFOS, PFNA, PFDA and PFUnA were in addition shown to affect both early initiation and later organization of polarization. Both compromised lumen formation and polarization have been suggested to play an important part in the disruption of an intact glandular structure, which has been linked to the earliest stages of epithelial breast cancer such as ductal carcinoma *in situ* (Leonard and Swain, 2004). Studies of alterations in polarization and luminal clearance in three-dimensional cultures can therefore provide valuable insight to how exposure by chemicals can affect DCIS development (Debnath et al., 2002).

DCIS has been shown to play an important role in the development of breast cancer, and of all *in situ* carcinoma of the breast diagnosed, 80 % is due to the development of DCIS (Siziopikou, 2013). The development of DCIS is mainly an early sign of breast cancer, characterized by morphological alterations, including increased proliferation, lack of polarization and luminal alterations (Al-Yusuf, 2005). Morphological alterations leading to

the development of DCIS do mainly occur in TDLU, linked to induced genetic modifications and changes in the micro-environment (Cichon et al., 2010). However, all DCIS will not develop into breast cancer, some will instead retrieve to atypical or intraductal hyperplasia (Bofin et al., 2004), characterized as a non-cancerous state that indicates increased risk of cancer (Purcell and Norris, 1998). Nonetheless, alterations in gland architecture can still indicate an increased risk of breast cancer development (Siziopikou, 2013). The indication is that, by resembling cells found in TDLU, MCF-10A replicates several aspects of the mammary gland and can therefore be used to study the potential effects of human exposure to PFCs in relation to breast cancer.

Increased risk of breast cancer due to PFCs has been observed in a study of human exposure of the Greenlandic Inuit (Bonefeld-Jorgensen et al., 2011). In Greenlandic Inuit study several legacy POPs and PFCs, including the compounds used in this study, were measured from serum samples from controls and breast cancer patients, which revealed a significant higher serum concentration of PFCs in breast cancer cases compared to controls. The mean sum of the PFCs measured was 8 ng/ml in serum from breast cancer patients and 5.3 ng/ml in the controls (Bonefeld-Jorgensen et al., 2011). These results demonstrate that human exposure to PFCs might increase the risk of breast cancer development, and that they potentially constitute a risk factor linked to breast cancer.

Although PFCs may represent a group of POPs that can contribute to the development of breast cancer, they only constitute a part of a combined mixture of POPs present in the human body (Porta et al., 2008). Some of these POPs, including Benzo[a]pyrene have been shown to alter acini architecture linked to DCIS and breast cancer development (Siriwardhana and Wang, 2008), which underlines that breast cancer development may be influenced by the mixture of POPs present in the human body, rather than individual PFCs. The presence of several POPs may also influence how these compounds affect biological functions, through additive, antagonistic or synergistic mechanisms (Ding et al., 2013). The potential interactions between POPs and the presence of several compounds in humans truly emphasize the complexity in revealing how these compounds may influence human and animal health. Studies of individual compounds are, nevertheless still important to fully characterize their potential effects and mechanisms of action.

4.3 Reasons for concern?

Mammary gland alterations initiated by puberty, pregnancy and lactation are strictly regulated by a number of specific hormones, protein interactions and cell-to-cell signals to ensure correct development and cell growth (Radisky et al., 2003). However, the number of alterations and factors involved during these phases also make the mammary gland susceptible to disruption of normal development by exogenous risk factors (Salehi et al., 2008, Macon and Fenton, 2013). Disruption of normal gland development has been reported to cause harmful effects later in life, including cancer (Fenton, 2006). Increased risk of breast cancer development linked to disruption of mammary gland includes compromised polarization, increased proliferation and inhibited lumen formation (Kumar et al., 2000).

The fact that PFCs used in the present study led to alterations in the cell cycle distribution and acini formation, indicate a potential disruptive effect on TDLU and thereby the mammary gland, which is a reason for concern in relation to breast cancer development. Alterations in TDLU structures have been linked to the development of DCIS (Sasano et al., 2010), classified as an early stage of cancer, being one of the four leading causes of breast cancer diagnosed in females (Kuerer et al., 2009). Additionally PFCs have been reported as a risk factor linked to breast cancer development (Bonefeld-Jorgensen et al., 2011).

However, when investigating effects of exposure using cell systems, it is important to consider if the *in vitro* effects are relevant and transferable to *in vivo* exposure. Although three-dimensional models mimic several aspects of tissue organization, it cannot fully replicate the *in vivo* situation. The same principle also applies for this present study of PFCs exposure on MCF-10A cells. In addition, *in vitro* exposures are mainly done for a short period with individual compounds at high concentrations, in contrast to human exposure, which is lifelong and to a complex mixture of compounds. However, the use of three-dimensional models and study of individual compounds can still be valuable for assessing potential hazardous human health effects due to exposure. Furthermore, studies of three-dimensional models have been reported provide important inside to human cell signaling, pathways and interaction (Weigelt and Bissell, 2008, Debnath and Brugge, 2005, O'Brien et al., 2002).

The exposure concentrations used in the present study are considerably higher than what has been measured in human breast milk and serum. Human serum in the Norwegian population has been measured to contain 32 ng/ml PFOS, 4.1 ng/ml PFOA, 1.1 ng/ml PFNA, 0.46 ng/ml PFDA and 0.71 ng/ml PFUnA (Haug et al., 2010), similar concentrations have also been measured in human breast milk in Sweden (Karrman et al., 2007). However, higher doses than those measured in the Norwegian population have been reported at in the Greenlandic Inuit, were PFOS concentrations in serum were 45.6 ng/ml. Moreover, since PFCs already have been linked to breast cancer development (Bonefeld-Jorgensen et al., 2011), and detected in both serum and breast milk, they could potentially constitute a part of the micro-environment of the breast, and therefore affect the risk of breast cancer.

This study revealed effects of exposure at the lowest doses, which indicate that lower doses than those used in the present study potentially could have an impact on acini morphology. Additionally, there is evidence in the literature that at environmental relevant exposure doses affected human health, including cancer development (Stahl et al., 2011, Bonefeld-Jorgensen et al., 2011).

5. Conclusion

The fact that PFCs are relevant in relation to breast cancer underlines the reasons for concern regarding these compounds, and the relevance of continued studies on their effects and mechanisms of action. Although *in vitro* studies cannot replace *in vivo* exposure scenarios, they do provide valuable insight to cellular responses and mechanisms of action (Pampaloni et al., 2007). Based on previous reports (Underwood et al., 2006), and the current study, I conclude that the MCF-10A human mammary epithelial cell line, particularly the three-dimensional cultures, is a valuable model for evaluating the effect of PFCs on morphological alterations *in vitro*.

The present data demonstrated a strong effect of PFOS, PFNA and PFDA exposure on acini morphology indicating that exposure to these compounds might be a risk factor involved in the development of breast cancer. Furthermore, the data indicate a more subtle effect of PFOA and PFUnA exposure, suggesting that these compounds were less toxic in the MCF-10A model. Further investigations are needed to document this conclusion and in addition, the mechanism behind the MCF-10A acini alterations caused by PFCs.

6. Future perspectives

Compromised lumen formation and polarization in MCF-10A acini indicate a potential effect of exposure to the test compounds on breast cancer development. However further studies of acini alterations, like integrin distribution for cell-cell and cell-ECM contact, should be included for more conclusive results. Further investigation of the vital dye results should also be considered to establish the effect of PFCs on the balance between apoptosis and proliferation. In addition, the mechanisms underlying the observed phenotypic alterations in MCF-10A acini induced by PFCs should be investigated.

Previous studies of phenotypic alterations in MCF-10A acini structures suggest overexpression of growth factor receptors and activation and inhibition of kinase activity (Pearson and Hunter, 2007, McNally et al., 2011). Therefore, the compromising effect on acini formation by PFCs might involve interactions between growth factor receptors and kinase activity, and these potential mechanistic interactions should be further investigated.

In addition, the present study investigated a limited spectrum of exposure doses, where exposure to PFOS, PFNA and PFDA caused morphological alterations in acini structure at the lowest doses. As a result, further studies into the lower dose range are indicated. The present study only reported effects of individual PFCs. In order to evaluate health risks linked to these compounds, more complex exposure scenarios and exposure models are indicated, including assessment of mixed exposures and use of *in vivo* exposure models.

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