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# Experimental evidence of a limited impact of new-generation perfluoroalkyl substance C6O4 on differentiating human dopaminergic neurons from induced pluripotent stem cells

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ABSTRACT

Perfluoroalkyl substances (PFASs) are persistent pollutants, raising concerns for human health. Legacy PFAS perfluoro-octanoic acid (PFOA) accumulate in brains of people at high environmental exposure, especially in areas enriched with dopaminergic neurons (DN). *In vitro* exposure to 10 ng/mL PFOA for 24 h was also associated with an altered molecular and functional phenotype of DN differentiated from human induced pluripotent stem cells (hiPSCs). Acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)– 1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1), known as C6O4, is a new generation PFAS proposed to have a safer profile. Here we investigated the effect of C6O4 exposure on the molecular phenotype of hiPSC-derived DN. Cells were exposed to C6O4 for 24 h, at the concentration of 10 ng/mL, at neuronal commitment (DP1), neuronal precursor (DP2) and the mature dopaminergic (DP3) phases of differentiation. Liquid-chromatography/mass-spectrometry showed negligible cell accumulation of C6O4 at each differentiation stage and by staining with Merocyanine-540 we observed unaltered cell membrane fluidity. Immunofluorescence showed that the expression of tyrosine hydroxylase (TH) and βIII-Tubulin was unaffected by the exposure to C6O4 at each differentiation phase (respectively: DP1, $p = 0.332$; DP2, $p = 0.623$; DP3, $p = 0.816$, with respect to control unexposed conditions). Exposure to C6O4 is presumed to have minor effects on cell molecular/functional phenotype of developing human DN cells, requiring confirm on *in vivo* models.

## 1. Introduction

In the last decades, great attention has been paid to disclose the possible effects of various chemicals that interfere with hormonal function, collectively known as endocrine-disrupting chemicals (EDCs), on human health. Among EDCs, poly- and per-fluorinated alkyl substances (PFAS) are raising health concerns worls-wide [1]. PFAS are a group of more than 4700 man-made chemicals, widely used for their unique chemical and physical stability. The most widespread PFAS in the environment and in human specimens are the legacy compounds perfluoro-octanoic acid (PFOA) and perfluoro-octane sulfonic acid (PFOS) among [2,3]. The exposure to PFAS raised concerns for their impact on human health since it has been associated, among the others, with metabolic derangements and impairment of the endocrine and nervous system, with particular regard to the hypothalamic-pituitary-gonadal (HPG) axis regulation. To date, very few studies have reported detectable amounts of PFAS in the human brain [4,5]. However, available studies in human populations described the possible impact of PFAS on neuro-endocrine and neuro-behavioral diseases, with different results depending on age, sex and outcome, with the increased risk for attention-deficit/hyperactivity disorder (ADHD) as one of the most consistent effects [6–11]. The toxico-dynamics of these chemicals on neuro-behavior is still unknown, but data from animal studies support the involvement of dopamine pathways. In a study on amphibian animal model, exposure to both PFOA and PFOS associated with a decreased content of dopamine in the

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central nervous system (CNS), without any major impairment of other neurotransmitters such as serotonin, norepinephrine, GABA, glutamate, or acetylcholine [12]. Accordingly, mice exposed to PFOS showed reduced levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the hippocampus [13].

These data are suggestive of the possible interference of PFAS with the dopaminergic network, thus representing a putative target of behavioral and cognitive alterations in humans. However, epidemiological data in humans are not consistent in both adult and young subjects with varying degrees of exposure to these contaminants. In addition, most studies focus on legacy PFAS such as PFOS and PFOA, whereas adverse effects of their new generation substitutes, such as acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)−1,3-dioxolan-4-yl)oxy)-ammonium salt (1:1), known as C6O4, are still lacking. At present, only one study from Coperchini et al. focused on the effect of C6O4 on a human model of thyroid cell lines, in terms of cell viability, ROS production and cell proliferation, but neuroendocrine-disrupting targets have not been evaluated yet [14]. In order to address the possible disrupting impact of this new generation PFAS on CNS dopamine network, in this study we aimed to evaluate the effect of C6O4 exposure on the differentiation and functional phenotype in a human *in vitro* model of human induced pluripotent stem cells (hiPSCs)-derived dopaminergic neurons.

## 2. Methods

### 2.1. Cell cultures

hiPS cells were a kind gift from Prof. Marta Trevisan (Department of Molecular Medicine DMM, University of Padua) and were obtained as previously described [15]. Cells at all culture stages were kept in a humidified incubator with 5 % CO2 at 37 °C. The hiPS cells differentiation into DNs protocol is a modified version from Borgs et al. as previously described [1,16]. At the day 6 of each differentiation stage, cells were exposed to C6O4 for 24 h at the concentration of 10 ng/mL.

Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)− 2,5-diphenyl-tetrazolium bromide (MTT) assays as previously described (Di Nisio et al., 2022). Lipid peroxidation was evaluated by a commercial kit involving the colorimetric detection of MDA-thiobarbituric acid adduct (ab118970, Abcam, Cambridge UK), as previously described [17].

### 2.2. LC-MS/MS analysis

Cell accumulation of C6O4 was measured through reversed-phase (RP) liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) as previously described [1]. After 24 h of exposure to PFOA, cells were detached by scraping and pellets were collected. Upon analysis, samples were defrosted at room temperature and extracted with 150 µl of methanol. Suspensions were finally sonicated for 10 min at room temperature, centrifuged at 13,000 rpm for 10 min and the resulting supernatants were used for chromatographic analysis. As internal standard, 200 µl of 13 C-PFOA 10 ng/mL were then added to each cell pellet. Samples were sonicated, centrifuged, dried as above mentioned and stored at − 20 °C until use. A system composed by a Varian binary pump, autosampler and diode array system equipped with a triple quadrupole model MS320 (Varian/Agilent Technologies, Santa Clara, CA, USA) was used. Stock solutions were prepared by diluting in methanol 13 C-PFOA and C6O4, to obtain final concentration of 500 ng/mL. The calibration curve was obtained mixing 50 µl of 500 ng/mL ISTD with different volumes (50, 25, 10, 5, 1 µl) of C6O4 standard solution in order to obtain different 13 C-PFOA/C6O4 quantity ratios for the calibration curve. PFAS were analyzed by LC-MS/MS, using electrospray ionization (ESI) source operating in negative ion mode. Needle voltage was 5000 V, drying gas temperature was 300 °C, drying gas pressure was 22 psi, nebulizer pressure was 55 psi, capillary voltage was set at 40, and CID gas was 1.5 mbar. Mass spectrum operating in negative ion mode and specific transition for 13 C-PFOA and C6O4 were obtained using MS software. Transition were as reported: 13 C-PFOA 417 > 372 and C6O4, 339 > 113.

### 2.3. Merocyanine staining and indirect immunofluorescence

Evaluation of plasma membrane fluidity was evaluated by Merocyanine 540 (MC540) probe as described elsewhere [1,18]. Cells were analyzed by BD FACScan System (Becton–Dickinson, Milano, Italy) and at least 10.000 events per session were recorded.

Indirect immunofluorescence was used to identify the expression of the enzyme tyrosine hydroxylase and the neuronal cytoskeleton protein βIII Tubulin. Cultured cells fixed with 4 % paraformaldehyde in PBS, were saturated with 5 % normal donkey serum/5 % bovine serum albumin in PBS and incubated with rabbit anti-TH (GeneTex, Irvine, CA, USA; 1:100) and mouse anti βIII-Tub (Abcam, Cambridge, UK 1:200). Primary immunoreaction was detected with the proper secondary antibody: goat anti-rabbit IgG (H+L)-Alexa Fluor Plus 488 conjugated and goat anti-mouse IgG (H+L)-Alexa Fluor Plus 633 conjugated (both from Thermo Fisher, Waltham, MA, USA). Control staining was carried out by using the secondary antibody alone. All stainings were performed in the same experimental session. Cells were finally washed in PBS and mounted in Mowiol (Sigma-Aldrich, Burlington, MA, USA). A Leica TCS SP5 confocal microscope was used to evaluate the expression of TH and βIII-Tub in the different phases of neurodifferentiation. To quantify the emitted fluorescence in gray-scale values, the Fiji ImageJ software was used. The integrated fluorescence density (I.F.D.) of each color channel was normalized to that of DAPI in order to harmonize expression data with cell density. The quantification analysis was carried out on 10 images acquired in triplicate at a magnification of 40X in mineral oil.

### 2.4. Statistical analysis

Results are reported as the mean value ± standard deviation derived from three independent experiments. Statistical analyses were performed with SPSS23 (SPSS, Chicago, IL, USA). Two-sided Student's t-test was used to compare two groups. When necessary, multiple comparisons were performed by the analysis of variance (ANOVA) with post-hoc Bonferroni's correction. Linear correlation analysis was performed through Pearson's coefficient. Results were considered significant for P values $< 0.05$.

## 3. Results

The possible accumulation of C6O4 in differentiating hiPSCs was evaluated by liquid chromatography/mass spectrometry (LC-MS/MS) as previously described [1]. In agreement with previous data on hiPSCs exposure to PFOA, cells were exposed to C6O4 10 ng/mL for 12, 24 and 48 h, at the end of each differentiation phase. In control samples (CTRL), in which exposure to C6O4 was omitted, levels of C6O4 were below the limit of detection (LOD=0.2 ng/mL) in any time point of observation. At 12 h of exposure to C6O4, no significant accumulation of the compound was observed (<LOD). At 24 h of exposure to C6O4, cells at the earliest phase of differentiation DP1 displayed a level of accumulation 0.6 ± 0.4 ng/mL that did not differ significantly from the corresponding control ($p = 0.1583$ *vs* CTRL). At the following phases of differentiation, DP2 and DP3, the observed cell levels of C6O4 were below the LOD. At 48 h of exposure, the observed cell levels of C6O4 at DP1 and DP2 were respectively 0.7 ± 0.6 ng/mL and 0.5 ± 0.4 ng/mL, showing non-significant difference compared to the corresponding CTRL (respectively: DP1 $p = 0.1301$ and DP2 $p = 0.1955$ *vs* CTRL).

In order to address any possible effect of C6O4 exposure on cell viability, hiPSCs were exposed to C6O4 10 ng/mL for 12, 24 and 48 h, at the end of each differentiation phase and evaluated by MTT assay. No statistically significant effect associated with the exposure to C6O4 was observed, compared to the corresponding unexposed control condition

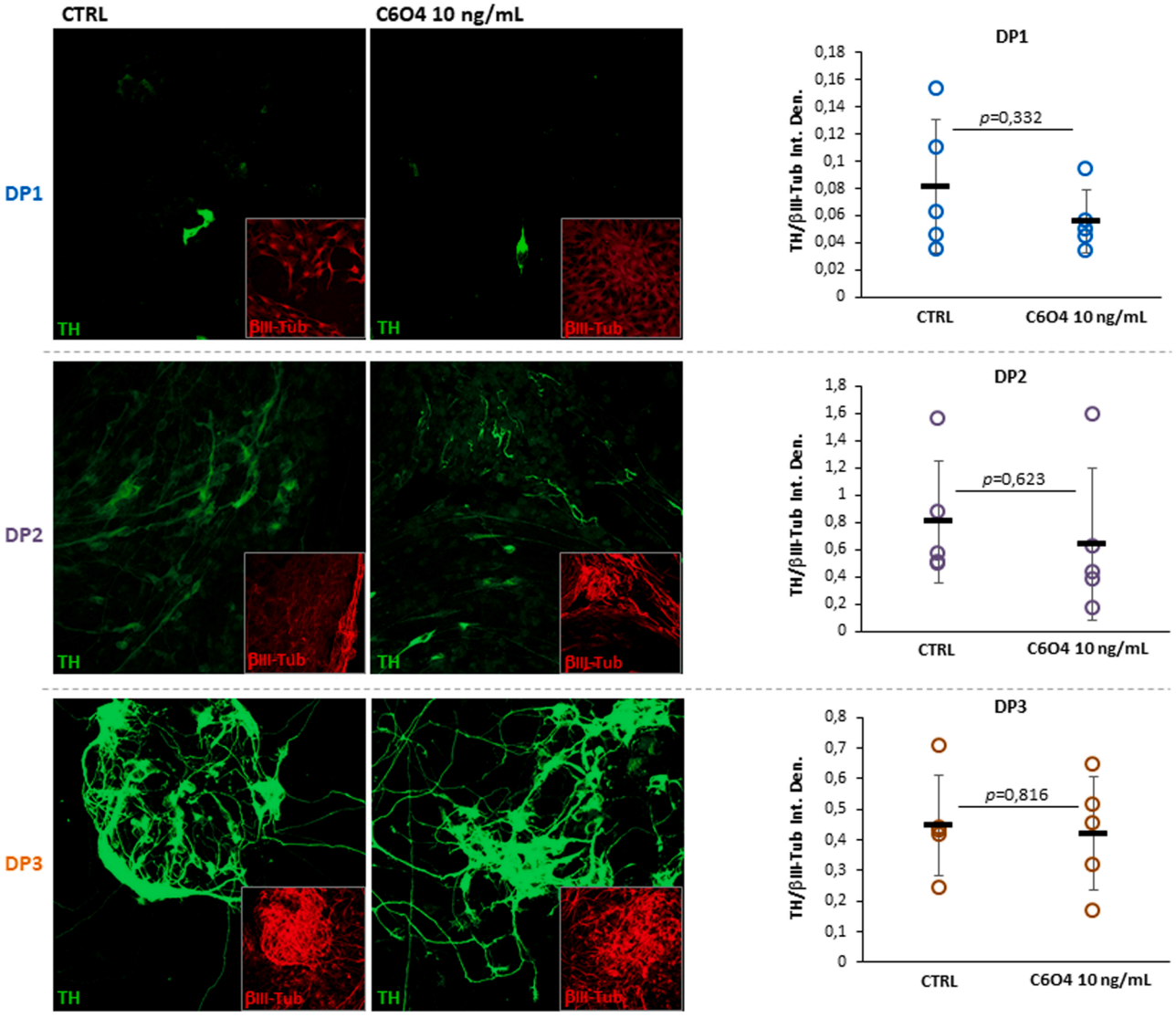


**Fig. 1.** Expression and quantification of Tyrosine Hydroxylase (TH, green) and βIII-Tubulin (βIII-Tub, red) at the three differentiation phases (DP1, DP2 and DP3) of human-induced pluripotential-stem cells (hiPScells) towards dopaminergic neurons in control conditions (CTRL) and upon 24 h exposure to 10 ng/mL of acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)– 1,3-dioxolan-4-yl)oxy)-ammonium salt (C6O4) as detailed in the methods section. TH expression is reported as TH/βIII-Tub fluorescence intensity ratio. Magnification 40X. p values of the comparison between CTRL and C6O4 conditions at each DP are reported.

(Supplemental Fig. S1A, all p values >0.05).

Since the different times of exposure were irrelevant for the purpose of cell accumulation, subsequent experiments were performed only at 24 h of exposure to C6O4 in order to provide a consistent comparison with previous data on PFOA [1].

Based on previous reports, showing major impairment of cell function associated with legacy PFAS exposure may rely on the alteration of membrane bilayer stability [19], we evaluated whether the observed altered function of differentiating dopaminergic neurons upon exposure to C6O4 was associated with an altered pattern of membrane's biophysical properties such as fluidity. To this end we used the bilayer fluidity-sensitive probe Merocyanin 540 (MC540) as previously described [18,20]. The exposure to C6O4 for 24 h at 10 ng/mL was not associated with significant variation in the mean fluorescence intensity of MC540 staining, a proxy of membrane fluidity, compared to unexposed control. Also, the evaluation of lipid peroxidation, assessed by malondialdehyde (MDA) production, showed that exposure to C6O4 10 ng/mL for 24 h was no associated with a significant variation compared to unexposed control (Supplemental Fig. S1B, all p values >0.05).

Finally, the effect of C6O4 exposure on the molecular phenotype of cultured human neuronal cells was evaluated through the expression tyrosine hydroxylase (TH), a marker of dopaminergic differentiation, by indirect immunofluorescence. The expression βIII Tubulin (βIII-Tub) was monitored as reference. Our data did not show any significant alteration of TH at neither DP1, DP2 nor DP3 (Fig. 1) after 24 h exposure to 10 ng/mL C6O4 compared to unexposed controls (all p values >0.05).

## 4. Discussion

PFAS are ubiquitous and highly persistent environmental contaminants, recognized to accumulate in the human body, raising concerns for their impact on human health: from the association with metabolic derangements to the role on altered cognitive behavior [1,21]. One of the most consistent clinical associations is the link between developmental exposure to legacy PFAS and increased risk for ADHD or its symptomatology [6].

A recent Norwegian study has reported that prenatal exposure to PFOA was associated with increased risk of autism spectrum disorders and ADHD in children [22]. Available studies suggest that symptoms of inattention and altered reward pathway in ADHD are linked to the

dysfunction of the dopamine system, such as the reduction in dopamine synaptic markers [23]. To this regard, a polymorphism of the dopamine transporter (DAT) gene has been associated with ADHD-like traits [24]. It can thus be speculated that the impact on the risk of developing ADHD by PFAS might rely on their interference on the dopaminergic system, particularly in the case of exposure during the most sensitive windows of CNS development in gestational life.

It should be noted that there are no available data describing serum or tissue levels of the new generation perfluoro-alkyl substance C6O4 so far. We thus decided to base our experimental setting on existing data on legacy PFAS, such as PFOA. To this regard, our previous findings, obtained on autopsy sampling, documented a particularly high concentration of PFOA in the brain areas richly innervated by dopaminergic neurons [1]. In addition, the exposure to PFOA at the concentration of 10 ng/mL was able to significantly impair the differentiation into dopaminergic neurons of human-induced pluripotency stem cells (hiPS). Specifically, compared to untreated cells, exposure to PFOA was associated with a reduced expression of TH and neurofilament heavy, both markers of dopaminergic commitment at DP2 phase. In addition, cells at DP3 phase exposed to PFOA showed a severe reduction in the expression of DAT, functionally involved in presynaptic dopamine uptake. These data are highly suggestive of a significant impact of PFOA exposure on neuronal development/function in both gestational and adult life. Accordingly, a significant deregulation of genes and biological pathways causally involved in the development of autism spectrum disorders were reported by a very recent study where a mixture of EDCs, including legacy PFAS such as PFOA and PFOS, on a cell model of fetal primary neural stem cells and on three-dimensional cortical brain organoids differentiated from human pluripotent stem cells [25]. On these bases, our study aimed to evaluate the possible effect of C6O4 on neuronal dopaminergic differentiation, at the same level of exposure previously used for PFOA. Incidentally, the concentration of 10 ng/mL has recently been used by Bruno et al. in the *in vitro* evaluation of the impact of C6O4 on renal transport function [26]. Differently from PFOA, we found that exposure to C6O4 is not associated with a significant cell accumulation of this compound in differentiating DN. In addition, a minimal impairment, if any, on cell membrane properties and on the molecular phenotype of differentiating dopaminergic neurons was detected. We surely account the use of the sole hiPS cell model as a study drawback, being primary dopaminergic neurons a suitable reference model [27].

However, these results add novel information about the possible interference of this new class of compounds on specific neuro-endocrine pathways, particularly in regard of the dopaminergic system, suggesting a different safety profile compared to legacy long-chain PFAS.

## 5. Conclusions

By the use of an *in vitro* model of human dopaminergic neurons, differentiated from human induced pluripotent stem cells, in this study we provide preliminary data supporting a minor effect on the cell molecular phenotype of these cells associated with the exposure to new generation PFAS molecule C6O4. Further data on *in vivo* models are required to confirm this hypothesis.

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## CRediT authorship contribution statement

**Andrea Di Nisio:** Writing – original draft. **Marta Trevisan:** Conceptualization, Writing – review & editing. **Stefano Dall'Acqua:** Investigation. **Micaela Pannella:** Investigation. **Claudia Pappalardo:** Investigation. **Alberto Ferlin:** Writing – original draft, Writing – review & editing. **Carlo Foresta:** Conceptualization, Funding acquisition. **Luca De Toni:** Conceptualization, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Carlo Foresta reports financial support was provided by Solvay Specialty Polymers Italy S.p.A.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.12.006.

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