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# Interference of C6O4 on platelet aggregation pathways: Cues on the new-generation of perfluoro-alkyl substance

Pietro Minuz [a,1], Luca De Toni [b,1], Stefano Dall'Acqua [c], Andrea Di Nisio [b], Iva Sabovic [b], Marco Castelli [a], Alessandra Meneguzzi [a], Carlo Foresta [b,*]

[a] Department of Medicine, Section of Internal Medicine C, University of Verona, Verona, Italy
[b] Department of Medicine and Unit of Andrology and Reproduction Medicine, University of Padova, Padova, Italy
[c] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy

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ABSTRACT

*Background:* Health concerns associated with the exposure to legacy perfluoro-alkyl substances (PFAS) led to the development of new-generation PFAS, such as C6O4. Here we investigated the possible effects of C6O4 on the platelet's activation profile, by incubating human platelets from healthy donors with C6O4 at different concentrations and evaluating the effects on activation, production and phenotype of platelets micro-particles (MPV) and aggregation under-flow. Based on the eventual platelet pro-aggregation profile detected, the preventive effect of acetylsalicylic acid (ASA) was also explored.

*Methods:* Adhesion-induced platelet aggregation of platelet rich plasma (PRP) under flow was evaluated on collagen-coated microchip at a shear stress of 10 Dyne. The turbidimetric method was used to investigate platelet aggregation. Finally, the *in vitro* generation of pro-coagulant MPV in PRP was evaluated by flow cytometry, as characterized by CD41 and annexin V positive events, under resting conditions and after stimulation with agonists at low shear stress.

*Results:* The generation of platelet aggregates under flow was significantly increased by the pretreatment of PRP with 100–200 ng/mL C6O4, compared to both the control condition and the experiment performed in presence of ASA. Arachidonic acid (AA), ADP and collagen induced an higher maximal aggregation, at turbidimetric evaluation, when PRP was pretreated with 100–500 ng/mL C6O4. In addition, PRP stimulated with AA also showed a steeper slope of the aggregation curve. The aggregation induced by the tested agonists was almost abolished by ASA. Finally, pretreatment with C6O4 increased the number of MPV in resting conditions and in presence of ADP and TRAP. ASA tended to reduce MPV generation.

*Conclusions:* Exposure to C6O4 associates with an increased platelet response to agonists, translating into a possible increased risk of cardiovascular events. Pending a further clarification on the toxicokinetics of this compound, our results claim the possible prophylactic use of ASA.

## 1. Introduction

Perfluoro-alkyl substances (PFAS) are synthetic compounds acknowledged for their chemical-physical stability and surfactant properties. Accordingly, they have been widely used in products such as fire-extinguisher foams, soil-extraction additives, house-hold detergents, films, waterproof clothing, and coatings for cookware (Kissa 2001; Moody and Field 2000). However, such characteristics give to PFAS a high environmental persistence and during the last two decades a large amount of studies showed a wide accumulation of these compounds in both environment and biota (Giesy and Kannan 2001; Lanza et al. 2017; de Vries et al. 2017; Navarro et al 2017). Particularly for two long-chain legacy PFAS, such as perfluoro-octanesulfonic acid (PFOS) and perfluoro-octanoic acid (PFOA), the bioaccumulation in humans due to environmental exposure has been associated with potential health issues. In fact, epidemiological studies focused on populations residing nearby production plants of PFOS and PFOA, showed an increased incidence of reproductive disorders, metabolic derangements

* Corresponding author at: Department of Medicine, Unit of Andrology and Reproductive Medicine, University of Padova, Via, Giustiniani, 2, 35128 Padova, Italy.
*E-mail address:* carlo.foresta@unipd.it (C. Foresta).
[1] The two authors equally contributed to the study.

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and cardiovascular mortality (Geiger et al 2014; Liu et al. 2018; Shankar et al. 2012; Huang et al. 2018; Di Nisio et al 2019; Di Nisio et al. 2020; Pitter et al. 2020). The possible pathogenic link between exposure to long-chain legacy PFAS and cardiovascular risk has been ascribed to the involvement in the atherosclerosis process (Lin et al. 2013; Lin et al. 2016). Less consensus has been reached regarding short-chain PFAS such as perfluorohexane-1-sulphonic acid (PFHxS) and undecafluorohexanoic acid (PFHxA), for which the association with ether coronary heart disease or known cardiovascular risk factors is weak or even absent (Honda-Kohmo et al. 2019; Zare Jeddi et al. 2021). To this regard, we recently showed that platelets membrane represents a preferential site for PFOA accumulation, leading to an impaired downstream signaling of platelet's activation and subsequent aggregation (De Toni et al. 2020).

On the base of these concerns about potential effects of legacy PFAS on human health, the production of PFOS and PFOA has been discontinued by primary manufacturers. To this regard, the fluorinated compound 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, has been claimed as a suitable substitute of legacy PFAS and has been approved by the European Chemical Agency (ECHA; https://echa.europa.eu/it/substance-information/-/substanceinfo/100.207.411) since 2012 because of its lower environmental and tissue persistence due to the novel cycle di-ether structure. The 2009 estimates regarding the production/importation of C6O4 in the European market are around 1–10 tons per year, nearly one tenth of those reported for PFOA (ECHA 2009; European Commission 2009). However, since the beginning of its production in Italy, appreciable concentrations of C6O4 were found in the river Po and very recent data of 2019 from the Regional Agency for the Prevention and Protection of the Environment in Veneto Region, reported a water concentration of C6O4 in water basin up to 100 ng/l (ARPAV 2019). In addition, positive detection of C6O4 has been observed in blood samples from workers employed in the production plants [http://www.google.it/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwi057SAscXrAhWCGewKHU8_Am0QFjAAegQIBRAB&url=http%3A%2F%2Frepository.regione.veneto.it%2Fpublic%2Fd2ec9f70f6cbc96bd4b3467dec922de7.php%3Flang%3Dit%26dl%3Dtrue&usg=AOvVaw32lCLF9tvPusI4teeUsOGX]. On these bases, despite epidemiological studies linking C6O4 exposure to health risk in humans are still not available, the early data dealing with the environmental exposure to the novel compound has raised some concerns. In this study we aimed to investigate the possible effects of C6O4 on platelet's activation profile. To this end, we incubated human platelets from healthy donors with C6O4 at different concentrations and evaluated the consequences on the activation, production and phenotype of platelets micro-particles and aggregation under-flow. The possible preventive use of acetyl-salicylic acid (ASA) on the eventual platelet pro-aggregation profile associated with C6O4 has also been explored.

## 2. Materials and methods

### 2.1. Chemicals

C6O4 standard compound was purchased from Wellington Laboratories, provided as 50 μg/mL methanol solution (CAS: 1190931–41-9; Southgate, Ontario, Canada). Equine tendon collagen (Collagen reagent Horm®) was purchased from Takeda (Lienz, Austria); acetylsalicylic acid (ASA), bovine serum albumin (BSA) were from Sigma Aldrich, Adenosine Diphosphate (ADP) and Thrombin Receptor Activator Peptide 6 (TRAP 6) were purchased from STAGO (Milan, Italy). PE-CD41, PE-Mouse IgG1 (IgG1 K,) and BD Trucount™ Tubes were purchased from Becton Dickinson (Milan, Italy). Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was from eBioscience (Thermo Fisher Scientific, Milan Italy). Arachidonic acid (AA) sodium salt and PPACK (trifluoroacetate salt) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Vena8 Fluoro + Biochips were purchased from Cellix Ldt (Dublin, Ireland).

### 2.2. Peripheral blood platelet sample preparation

This study was approved by the Institutional Ethics Committee of the Hospital of Padova, Italy, (protocol number 2208P and successive amendments). The investigation was performed according to the principles of the Declaration of Helsinki. Human whole blood was donated by 6 male healthy volunteers (mean age 43.7 ± 2.7 years), upon signing of the informed consent, who did not take any drugs in the previous two weeks, according to the guidelines of the ethical committee for clinical research of the Verona and Rovigo provinces and the principles of the Declaration of Helsinki. A clean puncture of an antecubital vein was performed with a 19-gauge needle (Safety-Multifly®-Set, Sarstedt, Nümbrecht, Germany) and blood collection was performed without applying venostasis. After discarding of the first 2–3 ml of blood, S-Monovette® tubes (Sarstedt) containing trisodium citrate 3.2% or PPACK100 μM were used as collection tubes and anticoagulant was immediately mixed with blood by gentle inversion. Platelet rich plasma (PRP) was obtained by centrifugation of blood at 180*g* at room temperature for 15 min while platelet poor plasma (PPP) was obtained by centrifugation 1 ml of PRP at 1600*g* for 5 min.

PRP was then incubated with or without increasing concentrations of C6O4 (1, 10, 100, 200 and 500 ng/mL for one and half hour at 37 °C. This explorative concentration range chosen for C6O4 was based on data available from previous studies on serum levels of other PFAS, particularly PFOA, observed in the general population (Zare Jeddi et al. 2021). In the negative control, where C6O4 was omitted, samples were equally added of 1:100 diluted methanol, in order to rule out any influence of the solvent. At the end of incubation PRP was tested for platelets aggregation, platelets-derived macrovesicles (PMV) generation and microfluidic analysis of adhesion-induced platelet aggregation.

In order to investigate the possible uptake of the compound by platelets, PRP incubated with C6O4 at the concentration of 500 ng/mL underwent to platelets isolation by centrifugation at 15,000*g* for 1 min.

In order to address the possible site of accumulation of C6O4, one milliliter of platelets underwent a fractioning process as previously described (Donovan et al. 2013). Briefly, platelets were resuspended in a hypotonic solution containing 100 μL citrate wash buffer and 900 μL deionized water, and kept on ice for 1 h. Following hypotonic lysis, the mixture was sonicated twice for five seconds at 20% amplitude to disrupt cell membranes and cytoskeletal fragments. Following sonication, the whole platelet homogenate was centrifuged at 1500*g* for 10 min to sediment any cellular debris. While supernatant was transferred to polycarbonate ultracentrifuge tube and centrifuged at 180,000*g* for one hour at 4 °C. The supernatant containing the soluble protein fraction was removed and saved and the resulting membrane enriched insoluble pellet was separated. All fractions were lyophilized and successively subjected to liquid chromatography-mass spectrometry as detailed below.

### 2.3. Liquid chromatography-mass spectrometry

C6O4 levels in platelets fraction were measured through reversed-phase (RP) liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) 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). Briefly, after dissolving the lyophilizate of platelet fractions and whole platelet in 200 ul of methanol, the mixtures were sonicated for 10 min and subsequently centrifuged at 3600*g* for 10 min. To test the analytical response and to optimize the calibration curve, standard solutions of C6O4 were prepared in appropriate range of concentration 0.15–50 μg/L. The solutions were analysed by LC-MS/MS, using electrospray (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 C6O4 were obtained using MS software. For C6O4, 339 > 113 Transition was used. The limit of Quantification was 0.5 ng/mL. For the chromatographic separation an Agilent XDB C-18 column was used (3 × 150 mm, 3,5 μm) and eluents were water 0.1% formic acid (A) and Acetonitrile (B) as previously described (Kaboré et al. 2018; Huset and M Barry 2018; Zhang et al. 2018). Gradient was starting with 90% A and in 0.5 min go to 50% A, then in 10 min 100% B and stay isocratic up to 20 min. Flow rate was 0.3 ml/min.

### 2.4. Docking analysis

The possible association between the C6O4 molecule and membrane lipids was theoretically investigated by docking methods. In the field of molecular modeling these methods lead to the prediction of the possible binding sites between molecules allowing the formation of a stable complex. The model of C6O4 ($C_6H_4F_9NO_6$) was estimated from the structure formula by using the software *MolView* (http://molview.org/; Smith 1995). A pre-equilibrated bilayer fragment containing 16 molecules of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was prepared by using *CHARMM-GUI Membrane Builder* (http://www.charmm-gui.org/?doc=input), a simulation preparation software (Lee et al. 2019). Both structures were prepared for docking by using the *DockPrep* module available in the *UCSF Chimera* molecular modeling software (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco; http://www.rbvi.ucsf.edu/chimera) and then minimized by using the *Yasara* software (http://www.yasara.org/minimizationserver.htm; Krieger et al. 2009). Flexible docking of C6O4 to the lipid bilayer surface was then performed by using the *AutoDock Vina* software (Trott and Olson 2010). The predicted molecular complexes were scored according to Gibbs free energy of binding and RMSD values. The docking solution with the best scores was considered and the abovementioned Yasara software was used to estimate the energy of the complex at equilibrium.

### 2.5. Platelet adhesion and aggregation under flow

Microfluidics experiments were performed on a Microfluidic Cellix platform (Cellix Ltd., Ireland) composed of a pump (Mirus Evo nano pump), an optic microscope (Leica DM IRB, objective magnification 20×, numerical aperture 0.30) equipped with EXi Blue fluorescence microscopy camera (1392 × 1040pixels, 800 Mb/s bandwidth capacity, 15 frames per seconds full resolution at 14 bits, 30 MHz, EXi Blue Q IMAGING) and Vena8 Fluoro$^+$ biochips (Cellix Ltd). Channels of the biochips were coated with 200 μg/mL equine tendon collagen (Horm collagen, Takeda) overnight at 4 °C in a humidified box (Philipose et al. 2010). To avoid unspecific binding the channels were blocked with 10 μg/ml bovine serum albumine (BSA, Sigma Aldrich) for 30 min at room temperature followed by washing steps with saline solution (NaCl 9 g/L).

PRP treated with increasing doses of C6O4 was diluted with physiological saline solution to obtain a final concentration of $1 \cdot 10^8$/ml platelets. Each specimen was then fluxed through the channels with a shear stress of 10 Dyne for 3 min.

Some experiments were also performed using PRP preincubated with ASA (100 μmol/L) to block cyclooxygenase-1 activity and prevent Thromboxane $A_2$ ($TXA_2$) generation.

During the experiment the temperature of the multichannel Biochip Vena8 Fluoro + was kept at 37 °C. After the perfusion, 20 images for each experiment were taken along the whole channels and analyzed with Duco Cell software (Cellix Ltd, Dublin, Ireland) which provides cell counting and analysis of morphological parameters of cells including area, diameter, perimeter, ellipticity, form-factor. Platelets adhesion on collagen surface was calculated and expressed as areas occupied by cells aggregates (Arbitrary Units, AU).

### 2.6. Platelet aggregation

Platelet aggregation was measured using PRP from sodium citrate anticoagulated blood on a four-channel aggregometer (APACT 4004 LABiTec, Germany) according to Born's turbidimetric method (Born and Cross 1963). The rate of platelet aggregation was determined by measuring the change in the optical density (transmitted light) of stirred PRP after addition of the aggregating agent to the aggregometer cuvette at 37 °C. The change in percentage of transmitted light (%T) was monitored for 5 min after the addition of the agonist to PRP. The 0% of aggregation was determined by transmitted light of PRP before addition of the agonist, while 100% aggregation was determined by transmitted light of a blank samples, ie plasma poor of platelets (PPP). The following agonist were used: 2.5 μmol/L ADP, 1.25 μg/mL Collagen, and 0.6 mmol/L AA. The concentrations of the agonists was preliminarily defined in order to obtain submaximal amplitude of platelet aggregation (80%). These experimental conditions are used in studies investigating drugs or compounds that may potentially amplify platelet response to aggregatory stimuli (Falcinelli et al. 2018).

All the experiments were performed both in the absence and presence of C6O4 (from 1 to 500 ng/mL), aggregation tests were also performed on PRP pretreated with C6O4 250 ng/ml and ASA 100 μmol/L.

For each aggregation test we considered the percentage of aggregation from 0 to 180 s for the calculation of slope and $EC_{50}$ and from 180 to 300 s to estimate the maximum aggregation (1 s intervals).

### 2.7. Release of platelets-derived microvesicles

The release of PMV was determined in PRP as described previously (Giacomazzi et al. 2016). PRP was obtained from 100 μmol/L PPACK anticoagulated blood in order to maintain physiological calcium concentration in plasma. PRP was treated with C6O4 250 ng/mL as described above. Aliquots (300 μL) of treated PRP were stimulated by incubation with various agonists for 30 min at room temperature under low shear stress conditions using GyroMiniTM Nutating Mixer (Labnet Int. Edison, NJ, USA). Saline solution (9 g/mL NaCl) was used instead of agonists for the resting conditions. Some experiment (in PRP treated with C6O4 250 ng/ml) were conducted also in presence of ASA 100 μmol/L in order to block platelet cyclooxygenase type 1 activity. The agonists used were the following: 7.5 μmol/L ADP, 10 μmol/L TRAP, 5 μg/mL collagen, 1.5 mmol/L AA. After incubation with agonists, PRP samples were centrifuge at 13.000*g* for 5 min at room temperature without break to obtain platelet-free plasma (PFP).

For PMV labeling, 40 μL of PFP was incubated in annexin V binding buffer in presence of annexin V-FITC and anti-human CD41-PE. A PE-Mouse IgG1 antibody was used as isotype control. After 30 min of incubation in the dark at room temperature, the reaction was diluted in annexin V binding buffer and transferred in BD Trucount™ Tubes (Becton Dickinson, Italy) which contain an established number of beads. PMV were finally analyzed by flow cytometry as CD41-PE and CD41-PE/annexin V-FITC-positive events in the PMV region which was determined by fluorescent beads of three diameters (0.5, 0.9 and 3 μm, Megamix, Stago, Biocytex, Marseille, France; Robert et al. 2009).

### 2.8. Statistical analysis

For statistical analysis, all data were analyzed with GraphPad Prism software v.5.03 (GraphPad Software, San Diego, CA, USA). Kolmogorov and Smirnov test was preliminary applied to evaluate normality of data distribution. Adhesion-induced platelet aggregation under flow was analyzed using one-way ANOVA followed by Tukey's test. Repeated measures one-way ANOVA and Tukey's test for post hoc analysis were applied to compare aggregation curves. $EC_{50}$ of platelets aggregation response was calculated by non-linear regression analysis. Friedman's

test and Dunn's test for post hoc analysis were used in the comparison of PMV release in each experimental condition. Data are expressed in the figures as individual data, or mean and standard error (Mean ± SEM) as indicated in figure legends. Data are expressed in the text as mean of differences with 95% confidence intervals or median and interquartile range. A statistical significance (P) value < 0.05 was set.

## 3. Results

### 3.1. Effects of C6O4 exposure on platelets membrane properties

On the base of the ability of legacy PFAS to stably interact with cell membranes (De Toni et al. 2020; Šabović, et al. 2020), we initially investigated the possible uptake of C6O4 by platelets. Accordingly, we incubated PRP, obtained from healthy unexposed subjects, with C6O4 at an exploratory concentration of 500 ng/mL and then we evaluated the possible content of the compound in isolated and washed platelets. Importantly, basal levels of C6O4 in platelets in control samples was 8.8 ± 3.8 pg/$10^6$ cells and incubation with the compound was associated with a significant increase of C6O4 levels to 263,0 ± 3,8 pg/$10^6$ cells (P < 0,001), suggesting that, as well as legacy PFAS, also C6O4 shows accumulation capabilities in human platelets. In order to disclose the possible accumulation site, cell membrane from platelets incubated with C6O4 were further isolated by ultracentrifugation (Fig. 1A). The LC-MS/MS analysis showed that the 74.2 ± 3.9% of the whole C6O4 platelet content was restricted to the plasma membrane fraction whilst cytosol fraction accounted for the 13.3 ± 0,6%. These data supported the hypothesis that, also for C6O4, plasma membrane represents the likely preferential site of accumulation in human platelets.

In agreement with previous reports, we investigated the possible consequences of C6O4 binding on the biophysical properties of cell membranes through a computational approach (De Toni et al. 2020). Accordingly, a molecular model of C6O4 was created and docking analysis to phosphatidylcholine (PC) was performed (Fig. 1B). Indeed, the Gibbs free energy (ΔG) changes value of −3.2 kcal/mol associated with the binding of C6O4 to PC suggested a possible stable interaction between the two molecules in a complex (PC-C6O4). In a first model of cell membrane including 50% of PC and 50% of

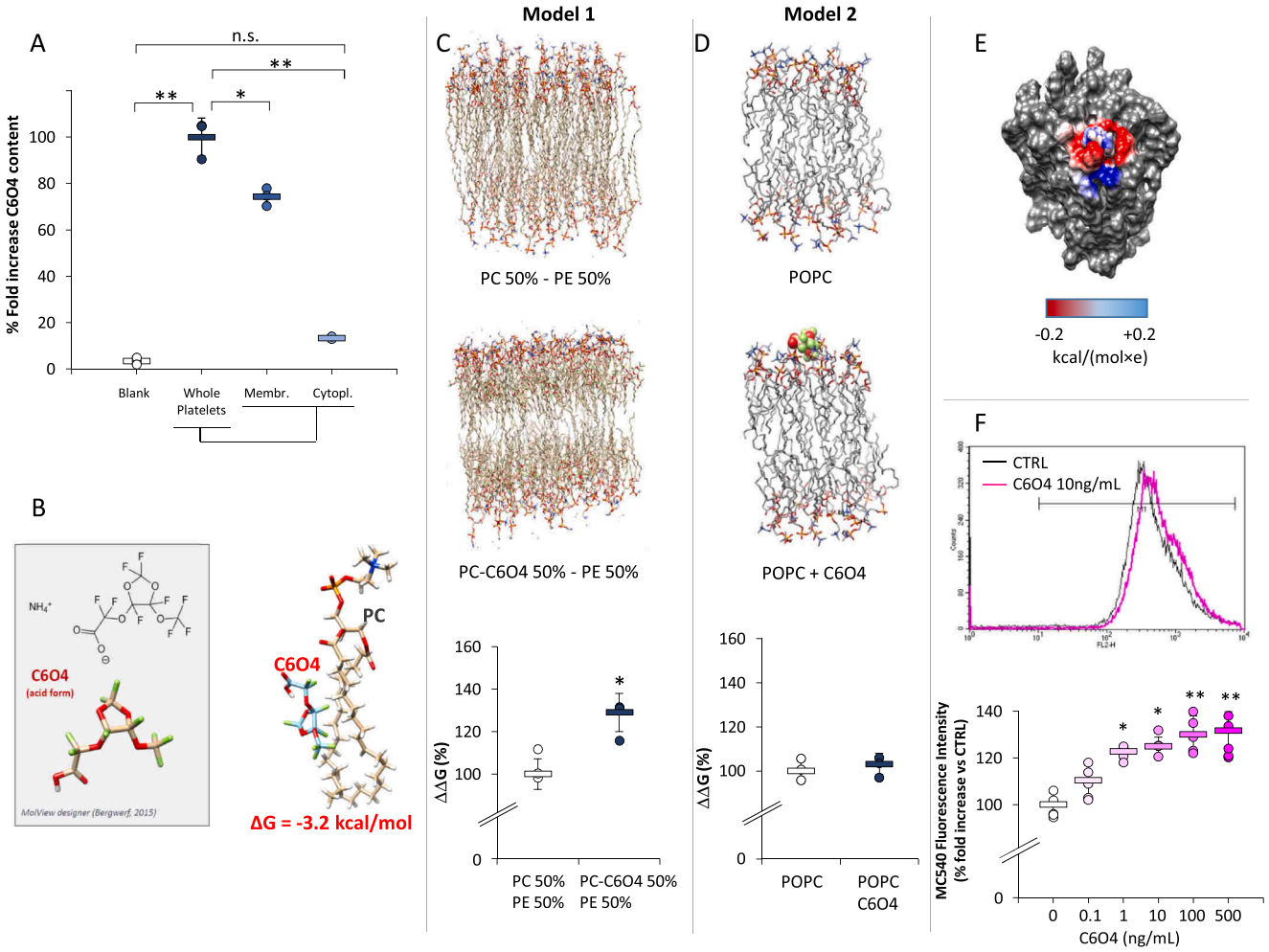


**Fig. 1.** *Accumulation analysis and docking interaction of C6O4 with platelets membrane* (A) Accumulation analysis of C6O4 in whole platelets, platelet's membranes and platelets cytoplasm, performed by liquid chromatography-mass spectrometry, as detailed in the method section, on three independent experiments. Significance: *=P < 0.05; **=P < 0,01 between the indicated conditions. (B) Representative images of C6O4 and the docking interaction between C6O4 and phosphatidylcholine (PC). The estimated Gibbs free energy (ΔG) changes is also reported. Panels (C) and (D) show the results of two interaction models of C6O4 with platelets membrane. In model I, the substitution of PC with the complex between PC and C6O4 (PC-C6O4) in a cell membrane system including 50% of PC and 50% of phosphatidylcholine (PE), was analyzed. In model 2, the flexible docking of C6O4 onto lipid bilayer surface was performed. The corresponding effects on the estimated ΔG changes of three independent simulations for each of the two models, are reported (ΔΔG). Significance: *=P < 0.05. Panel (E) shows the variation of the electric field closed to the binding site of C6O4 on membrane surface according to model 2. (F) Flow cytometry analysis of platelet's membrane fluidity by the staining fluorescence intensity of merocyanin 540 (MC540) after exposure to C6O4 at the indicated concentration. Data from 5 independent experiments are reported as percentage fold increase compared to control condition (CTRL) where C6O4 was omitted. Significance: *=P < 0.05; **=P < 0,01 *vs* (CTRL).

phosphatidylethanolamine (PE), the substitution of PC with the complex PC-C6O4 was associated with a ΔG increase by nearly 30% compared to the native composition, suggesting that the possible interaction of C6O4 with phospholipids may make the membrane more fluid or more easily destabilized (Fig. 1C). Considering the non-linear structure of C6O4, possibly hampering the insertion of the compound between the fatty acids chains of the lipid bilayer, a second model of interaction was created through a bilayer fragment containing 16 molecules of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) on whose surface a molecule of C6O4 was then affixed (Fig. 1D). Interestingly, despite a modest increase of the ΔG changes compared to the naïve composition was observed (~3%), the qualitative analysis of the electric potential on the membrane surface after binding of C6O4, showed a relevant variation of the electric field closed to the binding site (Fig. 1E), suggesting a major influence of the C6O4 exposure on the cell–cell surface interaction (Li et al. 2014). Based on the possible influence of C6O4 exposure on membrane bilayer stability, we evaluated whether this evidence translated into major effects on membrane's biophysical properties such as fluidity. To this end we used the bilayer fluidity-sensitive probe Merocyanin 540 as previously described (MC540; Buffone et al. 2006; Williamson et al. 1983; Langner and Hui 1993; Rathi et al. 2001). Interestingly, the mean fluorescence intensity of MC540 staining showed a significant and progressive increase following the incubation with C6O4 at concentrations ranging from 1 to 500 ng/mL, with an apparent saturation effect at concentration equal or greater than 100 ng/mL (Fig. 1E).

Taken together, these data are supportive of a major influence of C6O4 interaction with the platelet's membrane, resulting in the bilayer destabilization, increased fluidity and altered surface electrostatic charge.

### 3.2. Effects of C6O4 exposure on platelet adhesion and aggregation under flow

To assess possible effects of C6O4 on platelet adhesion and aggregation under flow, PRP was incubated for 30 min at 37 °C with C6O4 at the concentration 0, 100 or 200 ng/mL, in the presence or absence of ASA 100 μmol/L. Platelets were seeded on a microchip, pre-coated with collagen and then subjected to microfluidics analysis under predefined shear rate at 37 °C. Under these experimental conditions, we preliminarily evaluated the inter-assay and intra-assay variability (17.7% and 7.3%, respectively). All the experiments were performed testing, in parallel channels and in a single chip, two different conditions: PRP pre-incubated without C6O4 or ASA (control sample) and PRP pre-incubated with C6O4 and/or ASA. Data are expressed as the ratio between collagen-adhering platelet aggregates generated from treated PRP, and the untreated PRP.

As shown in Fig. 2 and video recordings in Supplemental materials S1A-F, platelets firmly adhering generate small aggregates upon the interaction and adherence to collagen fibers, which increase in size through the recruitment of flushed platelets. This process leads to aggregates, covering large areas in the micro-vessels, that can be quantified after flushing the system with saline solution. Using this methodological approach, we observed that the platelet aggregates were significantly increased by the pre-incubation with C6O4 100 ng/mL (+0.60 AU, 95% CI: +0.21 to +0.99 AU; $P < 0.05$ *vs* control) and 200

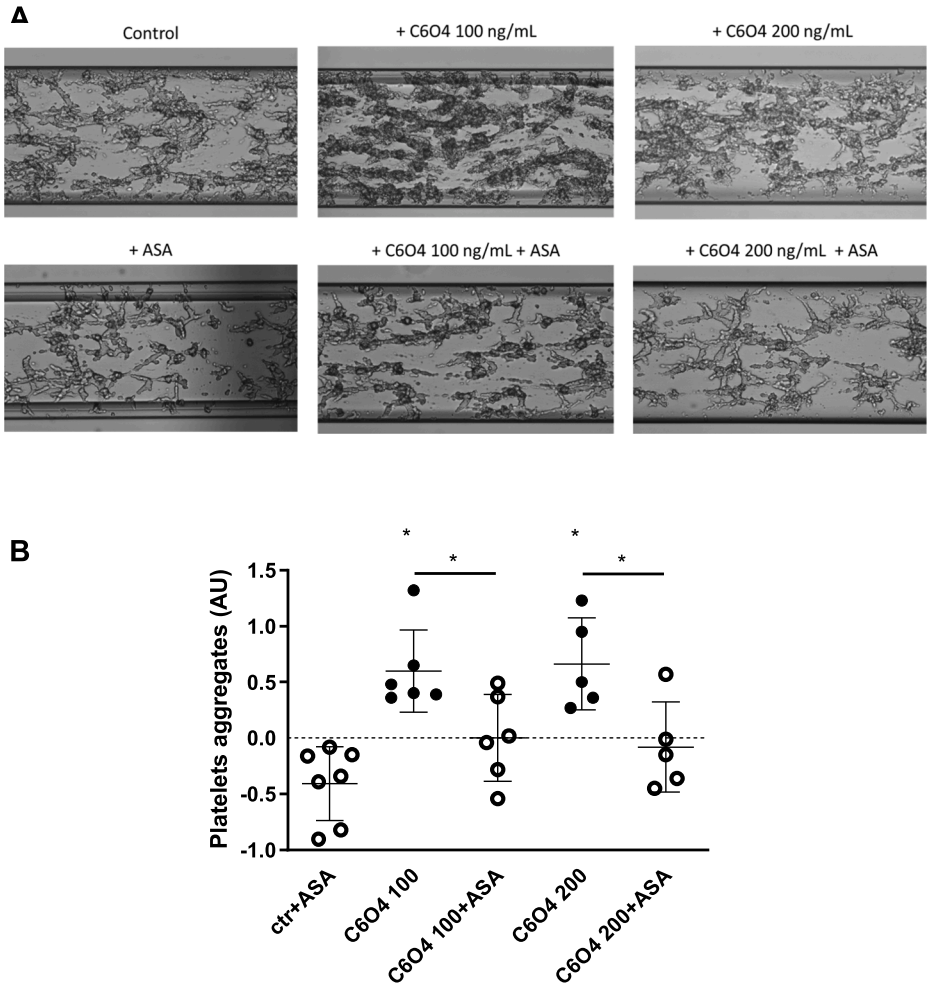


**Fig. 2.** *Microfludics analysis of platelet adhesion and aggregation* (A). Representative images showing platelet adhesion to immobilized collagen under different experimental conditions and platelet thrombus formation. Flowing in collagen-coated microchannels adhering platelets recruit circulating platelets to generate large stable aggregates. Prior to the analysis PRP was incubated for 30 min at 37 °C without or with 100 or 200 ng/mL C6O4, in the presence or absence of ASA 100 μmol/L. (B) Quantitative analysis of the area covered by adhering platelets under control conditions and in the presence of C6O4 with and without acetylsalicylic acid (ASA). The ratio of collagen-adhering platelet aggregates generated from treated PRP to aggregates from untreated PRP flowing at the same time in two parallel microchannel are calculated. Data are presented as individual values normalized versus control conditions representing platelet-covered area. Mean and standard deviation (Mean ± SD) are shown. One-way ANOVA followed by Tukey's test as post hoc analysis were applied. Significance: *=$P < 0.05$.

ng/mL (+0.66 AU, 95% CI: +0.15 to +1.17 AU; P < 0.05 *vs* control). Incubation of PRP with ASA 100 μmol/L significantly reduced platelets adhesion and aggregation under flow to immobilized collagen, both when PRP was not pretreated with C6O4 (-0.40 AU, 95% CI: −0.11 to −0.71 AU; P < 0.05) and when was treated with 100 ng/mL C6O4 (-0.60 AU, 95% CI: −0.11 to −1.08 AU; P < 0.05 or 200 ng/mL C6O4 (−0.74 AU, 95% CI: −0.06 to −1.48 AU; P < 0.05).

### 3.3. Effects of C6O4 exposure on platelets aggregation

The effects of C6O4, at concentration ranging from 100 to 500 ng/mL, on platelet aggregation were tested using PRP and different agonists using Born's turbidimetric method. Firstly, we tested the effects of pretreatment of PRP with 1, 10 and 100–500 ng/mL C6O4 on AA-induced platelet aggregation. As shown in Fig. 3A, a statistically significant increase in platelet response to AA was observed with 100–500 ng/mL C6O4 (0–300 sec: +8.5%; 95%CI: +5.1% to +11.8%). Indeed, exposure to C6O4 100–500 ng/mL accelerated platelet aggregation induced by AA, ($EC_{50}$ control conditions: 108.4 sec, 95% CI: 106.3–110.5 sec; $EC_{50}$ C6O4: 71.07 sec, 95% CI: 69.33–72.82 sec) and increased the maximum platelet aggregation (time lapse 180–300 sec: +1.9%, 95% CI: +1.2 to +2.2%; P < 0.0001 *vs* control condition). As shown in Fig. 3B and 3C, increased platelet response was observed comparing 1 ng/mL C6O4 (0–300 sec *vs* control: +11.6%, 95% CI: +5.8% to 17,47%, n = 5, P < 0.0001) and 10 ng/mL C6O4 (0–300 sec *vs* control: +10.1, 95% CI: +4.2% to 16.0%, n = 4, P < 0.0001) with statistically significant higher maximum aggregation. We did not observe any clear dose–response relationship to C6O4 at concentrations in the range of 1–500 ng/mL. Therefore, all the following experiments were performed using 100–500 ng/mL C6O4, in order to test its potential biological activity on platelet function. When PRP was tested in the presence of C6O4 and ASA 100 μmol/L, we observed a virtually complete inhibition of the platelet aggregation induced by AA, with statistically significant differences compared to both controls and C6O4 (respectively: −46.1, 95% CI: −42.8% to −49.5% and −54.6, 95% CI: −51.2% to −57.9%; Fig. 3A).

When aggregation was induced by ADP 2.5 μmol/L (Fig. 3D), we observed a larger amplitude of aggregation, testing PRP treated with C6O4 (n = 12), compared to matched control samples (n = 7) (+6.0%, 95% CI: +4.3% to + 7.7%, P < 0.0001). In particular, the slope of the curve was not altered by C6O4 ($EC_{50}$ control: 26.9 sec, 95% CI: 24.5 – 29.2 sec *vs* $EC_{50}$ C6O4: 26.9 sec, 95% CI: 24.9 to 28.8 sec) while the maximum platelet aggregation was increased (+8.5%, 95% CI: +8.0% to + 9.8%, P < 0.0001). A statistically significant reduction of platelet aggregation observed in presence of ASA 100 μmol/L compared with both control conditions (-31.1% 95% CI: −28.3 to −33.8, P < 0.0001) and with PRP treated with C6O4 (-37.1%, 95% CI: −34.3 to −39.2%; P < 0.0001).

Platelet aggregation induced by collagen 1.25 μg/mL (control n = 5) was significantly altered by 100–500 ng/mL C6O4 (+2.3%, 95% CI: 1.8% to + 2.8%, n = 7, Fig. 3E). While the slope of the curve was similar in untreated and C6O4-treated PRP ($EC_{50}$ control: 104.7 sec, 95% CI: 102.6 to 106.8 sec; $EC_{50}$ C6O4: 106.1 sec, 95% CI: 105.1 to 107.2 sec;), maximum platelet aggregation was increased in PRP pretreated with C6O4 compared to untreated PRP (+5.3%, 95% CI: +5.1% to +5.4%, P < 0.0001). Pretreatment with ASA 100 μmol/L (Fig. 3E) almost abolished collagen-induced aggregation either testing untreated (-68.6%, 95% CI: −68.2 to −68.9; P < 0.0001) or C6O4- treated PRP (73.8%, 95% CI: −73.5 to −74.2; P < 0.0001).

### 3.4. Effects of C6O4 exposure on platelets microvesicles release

The *in vitro* generation of pro-coagulant PMV was assessed measuring particles in the range of 0.1–1 μm expressing CD41 and binding annexin V (double positive PMV). The assay was performed applying a low intensity-shear stress to PRP, stimulated or not with a platelet agonist (Fig. 4). All the tested agonists increased PMV generation, particularly AA 1.5 mmol/L and collagen 5 μg/mL represented the strongest stimulus for *in vitro* PMV generation.

As shown in Fig. 4A, pretreatment of PRP with C6O4 250 ng/mL was associated with increase in pro-coagulant PMV from resting PRP in the absence of any platelet agonists. (median: 581 events, interquartile: 347–1030 events *vs* 1622 events, interquartile: 707 – 2022 events; P < 0.05). No significant difference in PMV release after stimulation with AA 1.5 mmol/L was observed between platelets exposed to C6O4 compared to naïve sample (n = 6) (median 2898 events, interquartile 1682–9403 events *vs* 3601 events, interquartile 2179–6281 events, P = n.s. Fig. 4B) or 5 μg/mL collagen (n = 5) (median 11,414 events; interquartile 6668–24393 events *vs* median 8514 events; interquartile 4406–20440 events; P = n.s. Fig. 4E). On the other hand, statistically significant differences were observed for platelets stimulated with either ADP (n = 6) (control: median 1032 events, interquartile 717–1732 events *vs* 250 ng/mL C6O4: median 2259 events, interquartile 912–3476 events; P < 0.05, Fig. 4C) and TRAP (n = 7) (control: median 1552 events, interquartile 1205–2103 events *vs* C6O4: median 2171 events, interquartile 1778–3672 events; P < 0.05, Fig. 4D). In the presence of ASA 100 μmol/L, a trend towards a reduced MPV generation was observed in all the tested conditions, including PRP not pretreated with C6O4.

## 4. Discussion

In this study, we provide evidence that C6O4, the novel substitute compound of legacy PFAS, is able to preferentially accumulate in the plasma membranes of human platelets, altering the major biophysical properties of this cell, such as fluidity and electrostatic charge distribution. These disrupting effects have detrimental consequences on both platelet aggregation profile, either in static or dynamic conditions, and on the extent of platelets microvesicles release. Even more importantly, we show that this pattern of altered platelet function is largely counteracted by the use of acetyl-salicylic acid as antiplatelet drug. To the best of our knowledge, this represents one of the very few studies aimed to address the possible health consequences associated with the environmental exposure to this novel compound.

By the use of a combined computational-experimental approach, we showed that the consequences of C6O4 interaction with platelets-plasma membrane largely overlaps with that of legacy PFAS, even taking into account the structural characteristics that differentiate the two classes of compounds, respectively cyclic and linear (De Toni et al. 2020).

To evaluate the potential effects of C6O4 on platelets, we performed three different sets of experiments, aimed to explore the three main functional activities of platelet in haemostasis and thrombosis: adhesion, aggregation and the release of microvesicles. Intriguingly, C6O4 enhanced all these platelet activities. In particular, we used a microfluidics apparatus to mimic the interaction between circulating platelets and vessels wall denuded of the endothelial layer, where PRP was flushed on collagen at laminar flow and constant flow rate, in analogy to what is observed in human arteries. Under these experimental conditions, platelet adhere to collagen through the engagement of integrins and further trigger platelet adhesion and platelet recruitment, generating platelet clot that covers collagen bed (Li et al. 2014). This process is dependent on the release of thromboxane $A_2$ ($TXA_2$) and ADP from activated platelets that amplify signals ensuing from integrin's ligation (Li et al. 2017; Ting et al. 2019). We observed that the whole process is significantly amplified by C6O4. It should be noted that, in order to test the hypothesis that C6O4 could have some biological effects on platelets, we tested relatively high concentrations of the chemical that, however, were in the range of those observed in humans exposed to PFAS (file:///C:/Users/utente/Downloads/Relazione.pdf; Girardi et al. 2018). Importantly, in our experimental setting, the effects of C6O4 on platelets could be detected when either resting platelets or agonists-stimulated platelets were used. In fact, increase in platelet aggregation was observed when platelets were stimulated with intermediate doses of AA

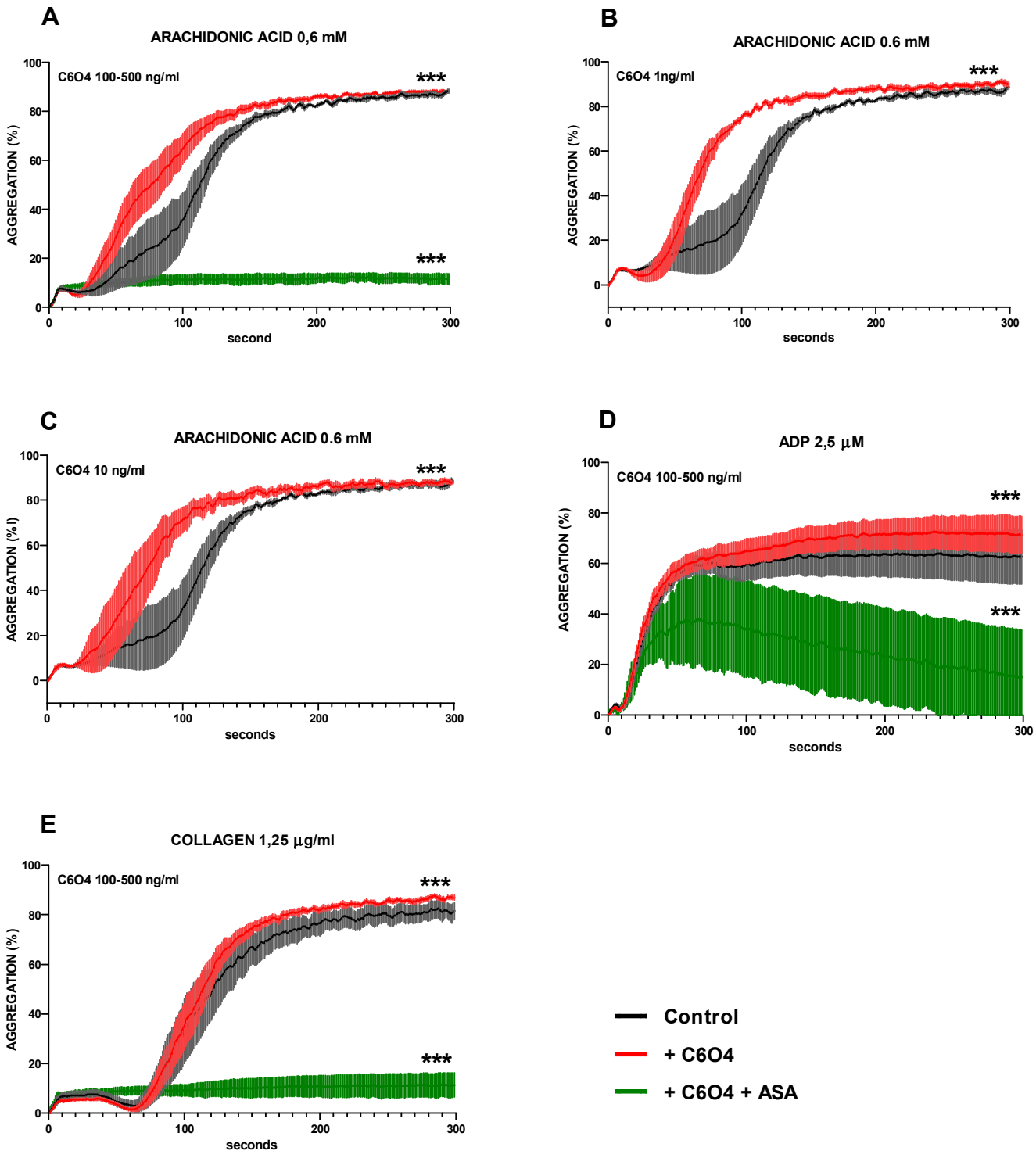


**Fig. 3.** *Agonist-stimulated platelet aggregation* Aggregation induced by different platelet agonists was analyzed using the turbidimetric method according to Born. (A) Platelet rich plasma (PRP), stimulated with Arachidonic Acid 0.6 mmol/L (AA), when pretreated with 100–500 ng/mL C6O4 (n = 12) shows a steeper slope of the aggregation curve compared to untreated PRP (n = 4) with higher maximum aggregation; Acetylsalicylic acid (ASA) 100 µmol/L (n = 3) blunts platelet aggregation of C6O4-treated and untreated PRP (n = 3). (B,C) Similarly, PRP pretreated with either 1 ng/ml C6O4 (n = 5) or 10 ng/ml C6O4 (n = 4) displays a steeper aggregation curve and increased maximum platelet aggregation compared with control conditions (n = 4) when stimulated with arachidonic acid 0.6 mmol/L. (D) Maximum aggregation induced by ADP 2.5 µmol/L is increased in platelets pretreated with 100–500 ng/mL C6O4 (n = 11) compared to control conditions (n = 7) and blunted by ASA (n = 3). (E) Platelet aggregation induced by collagen 1,25 µg/mL: pretreatment with C6O4 100–500 ng/mL (n = 8) shows increased maximum aggregation (from 180 to 300 s) compared to untreated platelets (n = 7). Aggregation is almost abolished in presence of ASA (n = 3). Data represent percentage of aggregation (%) recorded each second up to 300. Data are expressed as mean and standard error of the mean (Mean ± SEM). Repeated measures one-way ANOVA and Tukey's test as post hoc analysis were applied; Significance: ***=P < 0.0001.

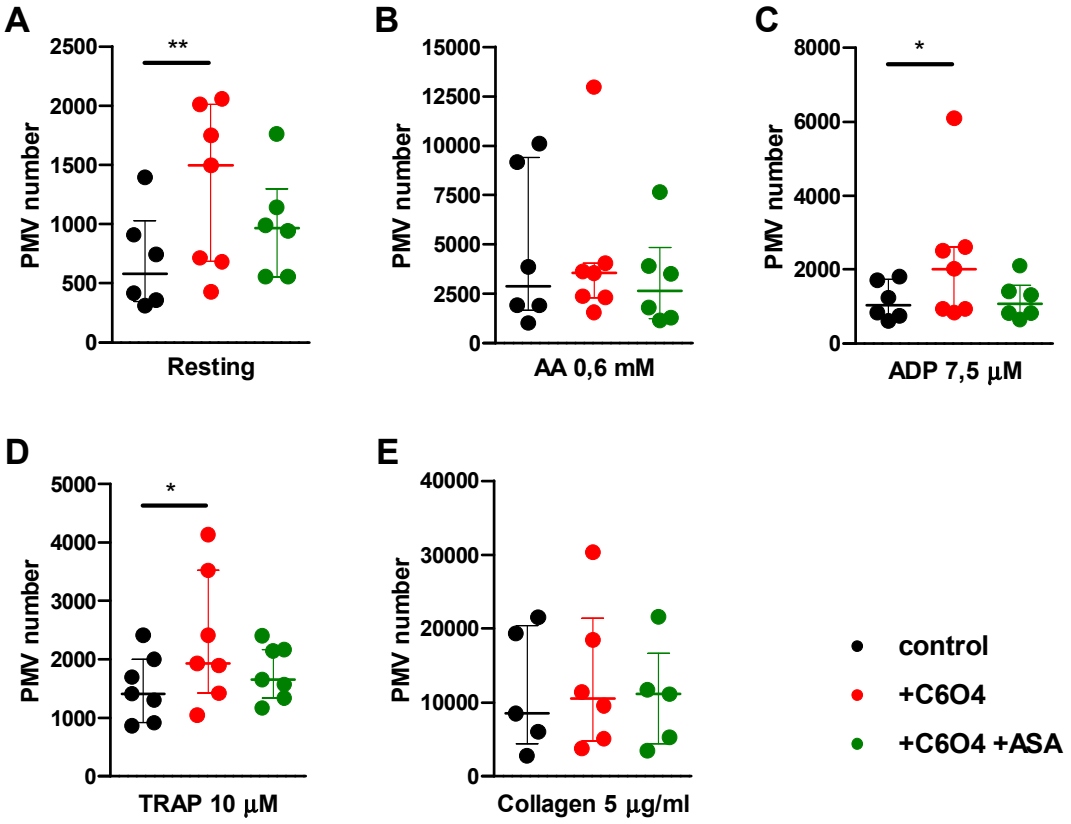


**Fig. 4.** *In vitro generation of procoagulant platelet-derived microvesicles In vitro* generation of procoagulant platelet-derived microvesicles (PMV) in unstimulated (A) platelet rich plasma (PRP) and in PRP stimulated with arachidonic acid 1.5 mmol/L (B), ADP 7.5 µmol/L (C), TRAP 10 µmol/L (D) and collagen 5 µg/mL (E). (A) The number of PMV generated in PRP pretreated with C6O4 250 ng/ml (n = 7) is compared with untreated PRP (n = 6) and ASA-treated PRP (n = 6). Increased PMV generation is observed in the presence of C6O4. (B, E) No significant difference in PMV release after stimulation respectively with arachidonic acid 1.5 mmol/L (n = 6) and collagen 5 µg/ml (n = 5) is observed between PRP treated with C6O4 and untreated PRP. (C,D) The number of procoagulant PMV generated in PRP pretreated with C6O4 250 ng/ml compared with untreated PRP are higher in platelets stimulated respectively with ADP (n = 6) and TRAP (n = 7). The presence of ASA 100 µmol/L, tends to reduce MPV generation but is not statistically significative. Friedman's test followed by Dunn's test as post hoc analysis were applied. Individual data are shown. Significance: *=P < 0.05; **=P < 0.01.

or ADP or a low concentration of collagen which induces an aggregation $TXA_2$-dependent, as shown by the effects of ASA (Gremmel et al. 2014). Concerning PMV, collagen and AA represent the strongest stimuli of release and their activity was not enhanced by C6O4. However, the experimental generation of platelet microvesicles requires the cooperative signals deriving from shear stress, a soluble agonist and the activation of an integrin (either aIIb/IIIa or the α2β1-GPVI complex; Giacomazzi et al. 2016; Burger et al. 2013). To this regard, exposure to C6O4 significantly amplified PMV release induced by shear stress alone and the stimulation with ADP and TRAP.

Further studies are then required to address the specific signaling pathways affected by the interference of this novel chemical. To this regard, other legacy PFAS substitutes in addition to C6O4, such as s perfluoro-2-propoxypropanoic acid also known as GenX, are now object of debate for their possible effects on human health (Coperchini et al. 2021). A straightforward comparison of their possible differential cardiovascular toxicity represents then a major issue to be addressed.

In all the experimental condition we tested, ASA blunted the effects of C6O4. This could be expected since its activity as inhibitor of $TXA_2$ generation, a key step in platelet adhesion, degranulation and partly in platelet aggregation and PMV release, consistent with the inhibitory effects observed also in the absence of C6O4 (Giacomazzi et al. 2016; Taus et al. 2019). The present observation defines the experimental conditions that could be applied in clinical studies aimed at defining any potential effect of C6O4 on platelets in vivo and the potential efficacy of acetylsalicylic acid in exposed subjects.

At present, only one study from Coperchini et al. showed no induction of necrosis or apoptosis exerted by C6O4 on two acknowledged human thyroid cell lines (Coperchini et al. 2020). Given such lack of scientific literature on the toxicology of C6O4, based on the only available information provided by the producer on the European Chemical Agency (ECHA; https://echa.europa.eu/it/substance-information/-/substanceinfo/100.207.411), this novel compound is expected to display a much shorter half-lives than legacy PFAS and reduced bioaccumulation, ruling out the major health concerns due to the long serum half-life observed for PFOA and PFOS in humans (Olsen et al. 2007). If this toxicological profile were to be confirmed by independent reports in the scientific literature, we support this new mechanism by which the new generation PFAS are able to obtain their biological effect on platelets through probable interaction with the platelet membrane. Of note, unlike the recognized mechanism of endocrine disruption, it does not necessarily require bioaccumulation in the body, but rather a relatively short-term interaction within the bloodstream that may be sufficient to interfere with platelet function.

In conclusion, the present results provide evidence of a biological effect of C6O4, showing increased platelet response to activatory stimuli. Whether this compound may exert its activity through a specific signaling pathway or mostly by altering membrane fluidity when a threshold concertation is reached needs to be further investigated. We did not observe a clear dose–response relationship in C4O6 activity on platelets.

## CRediT authorship contribution statement

**Pietro Minuz:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Luca De Toni:** Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Stefano Dall'Acqua:** Software, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Andrea Di Nisio:** Software, Validation, Formal analysis, Visualization. **Iva Sabovic:** Investigation. **Marco Castelli:** Investigation. **Alessandra Meneguzzi:** Formal analysis, Investigation, Writing - original draft. **Carlo Foresta:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

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

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## Appendix A. Supplementary material

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