Nordic Council of Ministers

Known and Unknown PFAS in Norwegian Adults:

Temporal Trends and Sources

Contents

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Abstract

This study investigated the amount and proportion of unrecognized fluorinated organic chemicals in humans and assessed exposure to these chemicals for two study days (T1 and T2 with 2 to 3 weeks apart) using the extractable organofluorine (EOF) and mass balance of EOF approaches. Samples were provided by the Norwegian Institute of Public Health (NIPH), Norway, through the EU project 'European Test and Risk Assessment Strategies for Mixtures' (EuroMix, 633172-2) and were collected from individuals living in the counties of Oslo and Akershus in Norway between September 2016 and November 2017. In the EuroMix study, a total of 143 subjects were studied at T1. Of these, 72 subjects were also studied at T2, forming a paired sample. In this specific investigation, samples from the EuroMix-study (Cohort 1) were used to examine how dietary habits and use of personal care products influence the proportion of unrecognized EOF in the blood. In a separate cohort, the potential contribution of fluorinated pharmaceutical (fluoxetine) to unrecognized fluorinated chemicals in humans was studied (Cohort 2). Results from Cohort 1 showed that up to 81% of the EOF was unrecognized (median 38%), and the amount of unrecognized EOF varied between individuals. In 36% of the paired samples collected 2 to 3 weeks apart, the EOF concentrations changed by more than 25%. No dietary habits or use of personal care products were associated with the proportion of unrecognized EOF in samples from T1. The proportion of unrecognized EOF in samples from T2 were associated with consumption of Potato/vegetables. Trifluoroacetic acid (TFA) was the dominant fluorinated organic chemical in the samples, with concentrations approximately twice that of PFOS. The use of fluorinated pharmaceuticals in Cohort 2 was associated with increased concentrations of both unrecognized EOF and TFA in the blood. The presence and concentrations of TFA and unrecognized EOF observed in this study warrants further investigation into its sources.

Background

In a report published in 2018 from OECD, more than 5,000 per- and polyfluoroalkyl substances (PFASs), a group of man-made compounds, are being used on the global market.[1] Due to the intrinsic chemical properties, they are very persistent in the environment and some of them are bioaccumulative. Three of them (e.g., perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and perfluorohexane sulfonic acid (PFHxS)) have been listed as persistent organic pollutants (POPs) under Stockholm Convention.[2] Several definitions of PFAS exist and their implications have been discussed.[3] Mostly adopted one in Europe is based on a report published by OECD in 2021,[4] which defines PFASs that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it).[5] Based on this definition, approximately 7 million compounds can be regarded as PFAS.[6] No matter which definitions are being referred to, less than 1% of these PFAS are included in human biomonitoring programs, suggesting that we might have overlooked human exposure to many new and unrecognized PFAS.[7]

Studies have shown declining PFOS and PFOA concentrations in human blood; however, a greater proportion of unrecognized PFAS were reported in human blood. [8] In a recent study, 130 whole blood samples from the Swedish general population were analyzed for extractable organofluorine (EOF) and 63 selected PFAS.[9] For the comprehensive PFAS assessment, analysis of EOF has shown to cover other unrecognized fluorinated organic chemicals compared to limited number of PFAS. Organofluorine mass balance analysis revealed that 60% (0–99%) of the EOF in female samples could not be explained by the 63 monitored PFAS; in males, 41% (0–93%) of the EOF was of unidentified origin.[10] The contribution of fluorinated pharmaceuticals to EOF has to be addressed as they might be co-extracted in the

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analysis and are present in the unrecognized EOF in the samples. A recent study on human sera samples in a Northern Norwegian cohort demonstrated that known PFAS through suspect screening only accounted for 2–4% of the EOF, fluorinated pharmaceuticals accounted for 0–63% of the EOF, and their contribution increased in recent years.[11] Both the diet and the use of personal care products have been identified to contribute to PFAS exposure in humans.[12] However, information on potential exposure pathways for unrecognized EOF exposure in humans is lacking. In another study, the EOF method was evaluated and proved to be an appropriate screening tool for identifying humans experiencing elevated PFAS exposure.[13] The average EOF concentration in the group with historical drinking water contamination from PFAS containing firefighting foam (Ronneby group) was 234 ng/mL F (<107–592 ng F/mL) vs 24.8 ng/mL F (17.6–37.8 ng F/mL) in the reference group. This large difference in the EOF concentrations between the two groups suggest that it is possible to identify samples with high PFAS exposure only using EOF data. Currently several Nordic countries (including Norway, Denmark, and Sweden) are in the lead to propose a general PFAS restriction in the EU. Before implementation of a future general PFAS restriction there is a need to establish a baseline of the concentrations of known PFAS and unrecognized EOF in human blood, facilitating time trend studies in the future.

Objectives of the study

- To establish an EOF concentration baseline in human blood prior to the implementation of a future general PFAS restriction.
- To conduct a mass balance analysis on EOF to evaluate the amounts and proportion of unrecognized EOF in human blood samples.
- To evaluate short-term changes in EOF concentrations in paired samples from the same individuals collected 2 to 3 weeks apart,) to provide insights into pharmacokinetics for biomonitoring purposes.
- To assess the impact of dietary habits and personal care product use on the proportion of unrecognized EOF in human samples.
- To evaluate the effect of fluorinated pharmaceuticals on EOF and unrecognized EOF concentrations in human blood samples.

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13. Aro, R.; Eriksson, U.; Kärrman, A.; Jakobsson, K.; Yeung, L. W. Y. Extractable Organofluorine Analysis: A Way to Screen for Elevated per- and Polyfluoroalkyl Substance Contamination in Humans? *Environ. Int.* **2022**, *159*, 107035. https://doi.org/10.1016/j.envint.2021.107035.

Method

Study subjects

Cohort 1 – EuroMix human blood samples. A total of 215 samples were analysed; 143 subjects were studied from T1. Of these, 72 subjects were also studied at T2, forming a paired sample which were collected 2 to 3 weeks apart. These samples were provided by the National Institute of Public Health (NIPH), Norway, via the EU project 'European Test and Risk Assessment Strategies for Mixtures' (EuroMix, 633172-2), funded by the H2020 programme.[14] The participants had completed a diary of their weighed food record and a cosmetic use diary on both study days. A food frequency questionnaire (FFQ) (only on the first day) and a questionnaire for socio-demographic and lifestyle characteristics (only first day). Details of the sample collection, data collection, registration and processing are published elsewhere.[15] The study was approved by the Regional Committees for Medical and Health Research Ethics (REK ID no. 2015/1868), and all participants provided written informed consent.

Cohort 2 – A total of 20 commercially available sera samples from the US were purchased from BioIVT. Ten serum samples were from individuals with records of taking fluoxetine at different dosages, while the other 10 were from individuals with no known use of fluorinated pharmaceuticals. These samples were analyzed for EOF and 25 selected PFAS and fluoxetine and its active metabolite (xeproxetine).

Target compounds

The 64 PFAS analyzed in Cohort 1 included perfluoroalkane sulfonic acids (PFSAs; C2-10, C12), perfluoroalkyl carboxylic acids (PFCAs, C2-C14, 16, 18), fluorotelomer carboxylic acids (FTCAs, 3:3, 5:3, 7:3), fluorotelomer unsaturated carboxylic acids (FTUCAs, 6:2, 8:2, 10:2), fluorotelomer sulfonic acids (FTSAs, 4:2, 6:2, 8:2), fluorotelomer phosphoric acid monoester (monoPAPs, 6:2, 8:2, 10:2), fluorotelomer phosphoric acid diester(diPAPs, 6:2, 8:2, 10:2), perfluorinated phosphoric acids (PFPAs, C6, C8, C10) and perfluorinated phosphinic acids (C6/C6, C6/C8, C8/C8), perfluoroalkane sulfonamides (C4, C6, C8, Methyl-C4, Methyl-C6, Methyl-C8, Ethyl-C8), fluoroalkane sulfonamidoacetic acid (C8, methyl-C8, ethyl-C8), perfluoroethylcyclohexane sulfonic acid (PFECHs),

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3H-perfluoro-3-[(3-methoxy-propoxy)propanoic acid (ADONA), hexafluoropropylene oxide dimer acid (GenX), chlorinated polyfluorinated ether sulfonic acids (Cl-PFESAs, 6:2, 8:2), perfluoro-3,6-dioxaheptanoic acid (3,6-O-PFHpA), perfluoro-4-oxapentanoic acid (PF4OPeA). The 25 PFAS studied in Cohort 2 included PFSAs (C2-10, C12), PFCAs (C2-C14), FOSA, 6:2 FTSA, and GenX.

Target fluorinated pharmaceuticals. Fluoxetine and seproxetine (one of the active metabolites of fluoxetine) were measured in sera samples of Cohort 2.

Sample pretreatment

Serum samples (0.5 mL for Cohort 2 and 2 mL for Cohort 1) were extracted at pH 4 using a modified ion-pair extraction method.[16] The sample extracts were split for EOF and targeted PFAS analyses. Additionally, the samples from Cohort 2 were underwent another round of extraction following the same procedure described above to capture fluoxetine and seproxetine, except that the pH was adjusted to 11 with ammonia solution before extraction. The sample extracts were also split for EOF, PFAS, fluoxetine and seproxetine analyses. The reason for extracting samples at pH 4 first was for comparison with other EOF studies in human blood samples with the same extraction method; the reason for extracting samples from Cohort 2 at pH 11 was that fluoxetine and seproxetine showed better recoveries at this pH; data are shown in the QA/QC section.

Instrumental analysis

The ultrashort-chain compounds (C2-C3 PFCAs and C1-C3 PFSAs) were measured using an supercritical fluid chromatography (SFC) system (Waters Ultra Performance Convergence Chromatograph, UPCC; Waters Corporation, Milford) coupled to a XEVO TQ-S (Waters Corporation) tandem mass spectrometer (MS/MS) using CO_2 and MeOH with 0.1% ammonia as mobile phases with a Torus DIOL analytical column (3.0 × 100 mm, 1.7 µm).[17] The majority of target PFAS (≥C4) were quantified using an Acquity UPLC coupled with a Xevo TQ-S MS/MS (both from Waters Corporation). Separation was achieved with a C18 BEH column (2.1 × 100 mm, 1.7 µm); mobile phases were MeOH and a 30/70 (v/v) mixture of MeOH and water with 2 mmol/L ammonium acetate and 5 mmol/L 1-methylpiperidine as additives.[18] Two novel PFAS (ADONA and hexafluoropropylene oxide-dimer acid (HFPO-DA)) were measured using a Waters Acquity UPLC system

16. Aro, R.; Eriksson, U.; Kärrman, A.; Yeung, L. W. Y. Organofluorine Mass Balance Analysis of Whole Blood Samples in Relation to Gender and Age. *Environ. Sci. Technol.* **2021**, *55* (19), 13142–13151. https://doi.org/10.1021/acs.est.1c04031.
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coupled with a XEVO TQ-S micro MS/MS; the column and mobile phase were the same as shown before.[19]

The EOF analysis was carried out on the combustion ion chromatography (CIC) system. Details of the method is available elsewhere.[20] Briefly, the CIC consisted of a Methrom IC (Herisau, Switzerland) connected to an autosampler and combustion module from Analytic Jena (Konrad-Zuse-Straße, Germany). An ion exchange column (Metrosep A Supp5-150/4) was used for separation of fluoride using an isocratic elution method with carbonate buffer with 64 mmol/L sodium carbonate and 20 mmol/L sodium bicarbonate, and the fluoride was measured with a conductivity detector.

Quality Assurance and Quality Control measures

- The sera samples in Cohorts 1 and 2 were stored at -80 ^{o}C at NIPH and BioIVT. Sera samples were packed with dry ice during the transportation to ORU, and they were stored in -20 ^{o}C in ORU before analysis.
- During every batch of sample extraction, two procedural blank consisting of ultrapure water and two sera blank consisting of fetal bovine serum (FBS) were included to keep track of possible contamination. If no detectable level of PFAS was found in the procedure blank, the method detection limit (MDL) was defined as the lowest point of calibration curve after taken into account of sample concentration factor. Otherwise, the MDL was defined as the average of the detectable concentrations plus three times of the standard deviation (SD). No detectable concentrations of PFAS were found in the procedure blanks (*n=24*). The MDLs for PFAS ranged from 0.01 to 3.5 ng/mL.
- Quantification of the 64 targeted PFAS and fluoxetine and seproxetine were performed based on internal calibration using corresponding mass-labelled standards; for those target analytes that did not have corresponding mass-labelled standards, the homologue closest in retention time was used for quantification.
- Detectable concentrations of EOF were observed in the procedure blanks of 1 batch of extraction which resulted in a higher MDL for that batch (8.9 ng F/mL). The MDL for EOF in the remaining batches was 7.5 ng F/mL. Detectable concentrations of EOF were found in the serum blank samples (*n= 22*) with an average of 19.2 ng F/mL with a SD of 1.9; two serum blank samples were lost during sample analysis because of instrumental error. No detectable concentrations of PFAS were observed in the serum blank and

19. Aro, R.; Eriksson, U.; Kärrman, A.; Yeung, L. W. Y. Organofluorine Mass Balance Analysis of Whole Blood Samples in Relation to Gender and Age. *Environ. Sci. Technol.* **2021**, *55* (19), 13142–13151. https://doi.org/10.1021/acs.est.1c04031.
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these detectable EOF results in the serum blank were used to evaluate repeatability.

- Before analyzing samples from Cohorts 1 and 2, a spike recovery test using FBS was performed to evaluate the recoveries of different PFAS. The recoveries ranged from 26% to 88%, with a maximum standard deviation (SD) of 21%. The spike recovery results for fluoxetine and seproxetine were 3% (SD: 1%) and 1% (SD: 1%), respectively, at pH 4, and 83% (SD: 5%) and 74% (SD: 5%), respectively, at pH 11.
- During each batch of extraction, two quality control (QC) samples (SRM1957 – non-fortified human serum) were also extracted to evaluate both the recovery and repeatability of the analysis. The recoveries of the certified PFAS ranged from 63% to 79%, with a maximum relative standard deviation (RSD) of 18%. The measured EOF in the SRM samples was 12.7 ng/mL F with an RSD of 5.3%.

Data treatment

Since the measured concentrations in this investigation were not recovery-corrected for both PFAS and EOF, the measured PFOS and PFOA concentrations were compared with previously published PFAS results from the EuroMix study from T1, where the data are presumed to be 100% as the samples had been recovery-corrected.[21] The comparison showed that the average recoveries of PFOS and PFOA in the current investigation were 48% (SD 17) and 47% (SD 16), respectively, when compared to the EuroMix study. Irrespectively of the recovery, all data were used for the mass balance analysis to estimate the amount of unrecognized EOF. However, samples with recoveries lower than 30% were excluded from the temporal analysis of EOF between T1 and T2. Further, mean estimated halving time for PFOS and PFOA have been found to be 5.3 years and 2.7 years,[22] respectively. Based on this, paired samples that showed changes in concentrations of PFOS or PFOA greater than 25% between T1 and T2 (13 pairs) were removed from the temporal analysis.

For fluorine mass balance analysis, the measured PFAS concentrations of all targeted analytes (ng/mL PFAS) were converted to respective fluoride-equivalent concentrations (ng F/mL) using the following formula:

$$C_F = n_F \times \frac{MW_F}{MW_{PFAS}} \times C_{PFAS}$$

21. Thépaut, E.; Dirven, H. a. a. M.; Haug, L. S.; Lindeman, B.; Poothong, S.; Andreassen, M.; Hjertholm, H.; Husøy, T. Per- and Polyfluoroalkyl Substances in Serum and Associations with Food Consumption and Use of Personal Care Products in the Norwegian Biomonitoring Study from the EU Project EuroMix. *Environ. Res.* **2021**, *195*, 110795. https://doi.org/10.1016/j.envres.2021.110795.
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where C_F is the concentration of fluoride (ng F/mL) coming from the compound, n_F is the number of fluorine atoms in an analyte molecule, MW_F is the molecular weight of fluorine, MW_{PFASi} is the molecular weight of the analyte *i*, and C_{PFASi} is the concentration of analyte *i* (ng/mL PFAS*i*). Similarly, the measured concentrations of fluoxetine and seproxetine were also converted into fluoride-equivalent concentration (ng F/mL) for comparison. When concentrations of analytes in the targeted analysis were below MDL, zero was assigned for them for mass balance analysis.

Investigation on factors that may affect the proportion of unrecognized PFAS in blood

Blood samples from the Cohort 1 were accompanied by information on sex, age, height, weight, dietary habits, and frequency of personal care product use. Information on dietary habits include information on amounts of different food types consumed such as bread, grain, cakes, potato, vegetables, fruit/berries, meat, fish, egg, dairy, cheese, butter/oil, sweets, beverages and other foods from previous publication. Information on the use of personal care products (PCP) included data on how many occasions the following items had been used during the 24h study periods for study T1 and T2: shower gel, shampoo, conditioner, deodorant, facial cleanser, facial moisturiser, body lotion, mouthwash, toothpaste, perfume, lip gloss/balm, foundation, hand cream, hair styling, eye make-up, rouge powder and hand soap. To explore if the proportion of unrecognized EOF in the samples was related to consumption of specific foods or use of certain PCPs, multivariable linear regressions (MLRs) were performed in the same manner as in the previous publication.[23] A three-step approach was used: Step 1) A univariate linear regression was performed between the proportion of unrecognized fluorinated chemicals as the dependent variable and each food or PCP group, age, and gender of the participants as independent variables to determine if there was a significant difference ($p < 0.05$) in consumption or use between males and females. The outcome decided if the MLR should be performed separately for males and females. Step 2) A second univariate linear regression between the proportion of unknowns as a dependent variable and each food or PCP group as the independent variable was performed to establish if the targeted food or PCP group was contributing to the proportion of unknowns. Age and gender were included as covariates. The food and PCP groups from these linear regressions with a p-value < 0.2 were included in the final MLR models for each chemical. Step 3) The MLR was performed with the proportion of unknowns as the dependent variable and by including the independent categorical variables with a p-value below 0.2 from step 2 as the independent variables.

23. Thépaut, E.; Dirven, H. a. a. M.; Haug, L. S.; Lindeman, B.; Poothong, S.; Andreassen, M.; Hjertholm, H.; Husøy, T. Per- and Polyfluoroalkyl Substances in Serum and Associations with Food Consumption and Use of Personal Care Products in the Norwegian Biomonitoring Study from the EU Project EuroMix. *Environ. Res.* **2021**, *195*, 110795. https://doi.org/10.1016/j.envres.2021.110795.

Results

A total of 215 samples from the Cohort 1 were analyzed for 64 targeted PFAS and EOF, while 20 samples from Cohort 2 were analyzed for 25 targeted PFAS, fluoxetine, seproxetine, and EOF; fewer PFAS were analyzed in Cohort 2 due to limited amount of volume of the samples to reach lower detection limit of other PFAS. The samples from this cohort were only focused on some legacy PFAS and GenX. Among the 215 samples from Cohort 1, 143 from T1 and 72 were paired samples from the same individuals at T2, which were collected 2-3 weeks apart.

Concentrations of target PFAS and EOF

i. Cohort 1- samples from the EuroMix study. Of the 64 PFAS analyzed, 20 showed detectable concentrations. Seven PFAS were detected in all 215 samples, with the highest median concentrations in the following order: TFA (6.75 ng/mL), PFOS (2.54 ng/mL), PFHxS (0.84 ng/mL), PFOA (0.75 ng/mL), PFNA (0.29 ng/mL), PFDA (0.11 ng/mL), and PFHpS (0.06 ng/mL). The reported PFAS concentrations were lower than those in an earlier study reporting PFAS in the same individuals.[24] This was expected as the PFAS concentrations measured in the current investigation were not recovery-corrected. As shown in the QA/QC section, the average recoveries of PFOS and PFOA in the current investigation were 48% (SD 17) and 47% (SD 16) when compared to the EuroMix study. For the recovery corrected human exposure data, please refer to the earlier study.[25] Regarding EOF analysis, a total of 164 samples showed detectable concentrations (7.6–32 ng F/mL, median: 14.6 ng F/mL).

ii. Cohort 2 - Samples were purchased commercially. These samples were first extracted at pH 4 and then pH 11; the extracts from pH 4 and pH 11 were analyzed using LC-MS separately. At pH 4, 14 of the 25 PFAS analysed had detectable concentrations. Seven PFAS showed detection frequency over 80% with the median concentrations in the following order: TFA (15.1 ng/mL), followed by PFOS (1.82 ng/mL), PFOA (0.67 ng/mL), PFHxS (0.51 ng/mL), PFNA (0.19 ng/mL), PFDA (0.08 ng/mL), and PFUnDA (0.03 ng/mL). No significant differences were observed between those taking medications and those who did not (Mann-Whitney U test, $p>0.05$), except for TFA.

24. Thépaut, E.; Dirven, H. a. a. M.; Haug, L. S.; Lindeman, B.; Poothong, S.; Andreassen, M.; Hjertholm, H.; Husøy, T. Per- and Polyfluoroalkyl Substances in Serum and Associations with Food Consumption and Use of Personal Care Products in the Norwegian Biomonitoring Study from the EU Project EuroMix. *Environ. Res.* **2021**, *195*, 110795. https://doi.org/10.1016/j.envres.2021.110795.
25. Thépaut, E.; Dirven, H. a. a. M.; Haug, L. S.; Lindeman, B.; Poothong, S.; Andreassen, M.; Hjertholm, H.; Husøy, T. Per- and Polyfluoroalkyl Substances in Serum and Associations with Food Consumption and Use of Personal Care Products in the Norwegian Biomonitoring Study from the EU Project EuroMix. *Environ. Res.* **2021**, *195*, 110795. https://doi.org/10.1016/j.envres.2021.110795.

Detectable concentrations of fluoxetine and seproxetine were only observed in the subjects who had taken fluoxetine ranging from 0.03–6.95 ng/mL and <MDL – 0.48 ng/mL, respectively. On the other hand, at pH 11, no detectable concentrations of target PFAS were observed. However, at pH 11 higher concentrations of fluoxetine and seproxetine were observed in the subjects who had taken fluoxetine ranging 1.26–218 ng/mL and 16.0–92.5 ng/mL, respectively. Regarding EOF analysis, elevated concentrations of EOF were observed in the subjects where they had taken fluoxetine when compared to the control group (4 out of 10 subjects without taking fluoxetine had detectable EOF concentrations). At pH 4, the fluoxetine group showed the median of 140 ng F/mL versus the control group of <8 ng F/mL; significant higher EOF concentrations were observed in the fluoxetine group (Mann-Whitney U test, $p<0.05$, Figure 1). Two elevated EOF concentrations in the control group were due to significant levels of 6:2 FTSA and PFHxS in the samples, respectively. Detectable EOF concentrations were only observed in the fluoxetine group at pH 11 (median: 28.4 ng F/mL).

Mass balance analysis of extractable organofluorine (EOF)

Mass balance analysis of EOF revealed that the target PFAS accounted for varying proportions of EOF in the samples, ranging from 5.8 % to being fully accountable in the EuroMix samples, as well as in samples from subjects who either had or had not taken fluoxetine or other fluorinated drugs (Figure 2). An important finding from this investigation is that the contribution of target PFAS drops considerably if TFA is not included in the mass balance analysis from median of 62% to 29%, indicating that TFA is the most abundant PFAS in this study. In Cohort 2, the mass balance drops from 14% to 2% among those who had taken fluoxetine, and from 58% to 27% among those who had not taken fluoxetine when TFA is excluded in the mass balance analysis. For the samples treated at pH 11, the only detectable compounds were either fluoxetine or/and seproxetine from the subjects who had taken fluoxetine.

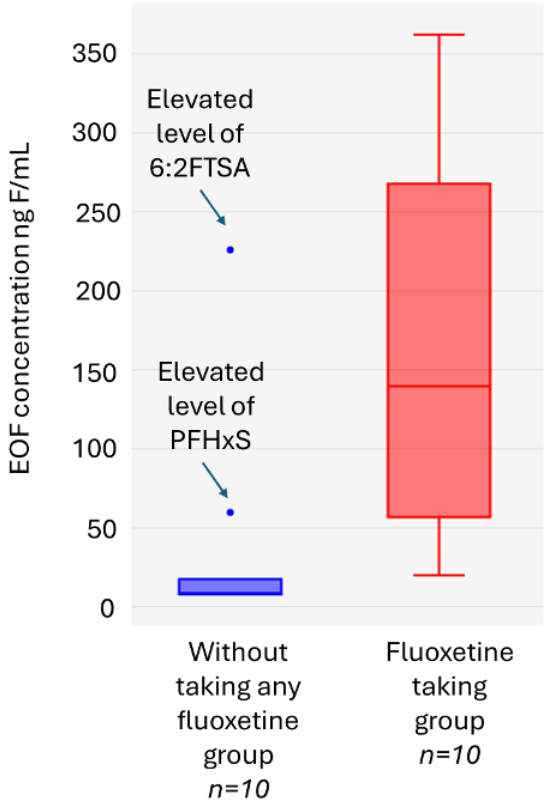

Figure 1. EOF concentrations (ng F/mL) in subjects without taking fluoxetine* and subjects taking fluoxetine of cohort 2. (*When EOF is below MDL, MDL was used; significant higher EOF concentration was observed in the fluoxetine group)

	min	max	median	average	SD	detection frequency
PFHpA	0.02	0.10				51
PFOA	**0.18**	**3.99**	**0.75**	**0.84**	**0.45**	**100**
PFNA	**0.08**	**1.41**	**0.30**	**0.35**	**0.21**	**100**
PFDA	**0.03**	**0.51**	**0.12**	**0.14**	**0.09**	**100**
PFUnDA	0.01	0.57	0.11	0.14	0.11	99
PFDoDA	0.02	0.07				27
PFTrDA	0.01	0.19				72
PFTDA	0.02	0.03				6
PFBS	0.010	0.03				8
PFHxS	**0.26**	**2.721**	**0.84**	**0.90**	**0.41**	**100**
PFHpS	**0.01**	**0.37**	**0.06**	**0.08**	**0.06**	**100**
L-PFOS	**0.30**	**8.36**	**1.65**	**2.09**	**1.61**	**100**
BrPFOS	**0.17**	**5.13**	**0.89**	**1.25**	**0.95**	**100**
6:2 FTSA	0.01	0.12				2
8:2 FTSA	0.01	0.08				46
TFA	**2.69**	**16.0**	**6.75**	**6.91**	**2.34**	**100**
PFPrA	0.18	0.59				22
FOSAA	0.02	0.05				4
MeFOSAA	0.01	0.22				22
EtFOSAA	0.02	0.03				1
FOSA	0.01	0.06	0.02	0.02	0.01	80

Table 1. Target* PFAS concentrations (ng/mL) and detection frequency (%) in samples from Cohort 1, the EuroMix study

*Reported concentrations are not recovery-corrected. Recovery-corrected concentrations, except for TFA and PFPrA, are provided in Thepaut et al. 2021.[26]

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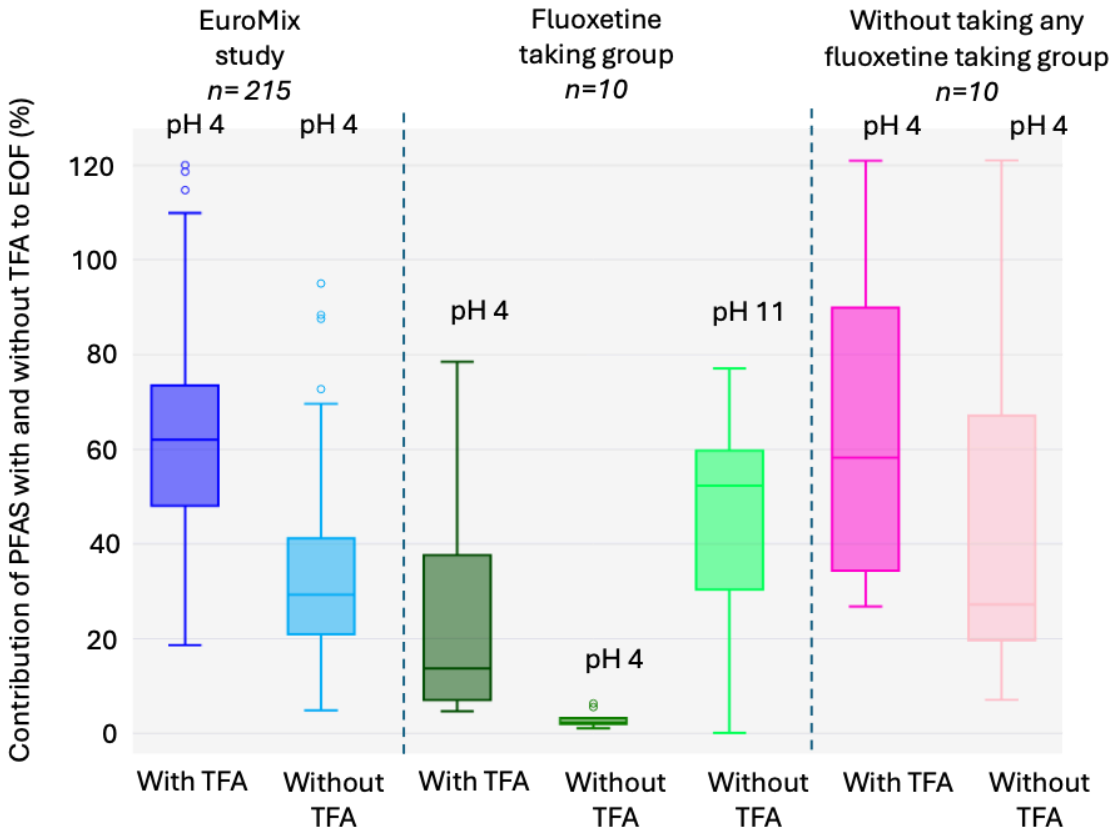

Figure 2. Percentage of target PFAS to EOF (%) in the sera samples

Note: When EOF was below MDL, value of MDL was used for EOF for calculating the percentage of PFAS to EOF. Since no TFA was detected in the fluoxetine group extracted at pH11, the graph is not shown.

Temporal changes of EOF and TFA in Cohort 1 between T1 and T2 (2–3 weeks between sample collections)

Table 2 summarizes the results of temporal analysis of EOF between paired samples collected with a 2 to 3 weeks time interval. Almost 50% of the samples did not show any significant changes (less than 25%) in EOF between T1 and T2; 31% showed a significant decrease with a maximum decrease of EOF of 54%; while only 5% showed significant increase with the maximum increase of 26%. In 15% of the cases, the EOF at T1 and T2 were below MDLs, for which we cannot tell if there would be any significant changes (25%) in EOF as undetermined. Eight pairs of samples showed 25% of increase of TFA concentration, while only 1 pair showed a decrease of 25% of TFA concentration. Only 3 pairs showed significant changes in both EOF and TFA.

Description	% of the pair samples (*n=59*)
Undetermined (both values between T1 and T2 were below MDL)	15
No difference (differences between T1 and T2 were below 25%)	49
Increase (25% increases between T1 and T2)	5
Decrease (min: 25% and max 54%) (25% decreases between T1 and T2)	31

Table 2. Changes in EOF between T1 and T2 (sample collection 2–3 weeks apart)

Investigation on factors that may be associated with the proportion of unknown fluorinated chemicals in blood

No dietary habits or use of personal care products (*p=0.139*) were associated with the proportion of unknown fluorinated chemicals in samples T1. However, the proportion of unknown fluorinated chemicals in samples from T2 were associated with consumption of Potato/vegetables (*p=0.0287*).

Discussion

Comparison of EOF concentrations of the Cohort 1 samples with other studies

A recent study[27] from Tromsø, another Norwegian region, reported EOF in serum samples ranging from 12.6 to 45.3 ng F/mL in samples from 1986, 2007, and 2015. Specifically, in 2015, EOF concentrations ranged from 12.6 to 22.6, with a median of 18.5 ng F/mL. Miaz and co-workers[28] reported a geometric mean of approximately 30 ng F/mL in pooled serum samples from first-time mothers in Sweden collected in 2015. Aro and co-workers[29] reported detectable concentrations of EOF in human whole blood collected between 2017 and 2018 from Sweden in the range between 0.51–48.7 ng F/mL. The average EOF concentrations in plasma samples from German university students in Munster, collected between 1982 and 2009, ranged from 14.5 to 39.3 ng F/mL. In general, different methods have different extraction efficiency for different PFAS,[30] thus a direct comparison of EOF concentrations may be challenging. The extraction methods and sample matrices (whole blood, plasma, and serum) differed between the present study with samples from the Oslo area (Cohort 1) and the one from Tromsø. However, the EOF concentrations in the samples from Cohort 1 (<7.5–32 ng F/mL, median: 14.6 ng F/mL) were similar to those observed in Tromsø (12.6 to 22.6 ng F/mL, median: 18.5 ng F/mL) collected around the same time.[31]

Mass balance analysis of EOF

Similar to the results from other studies, our mass balance analysis of EOF revealed that quantifiable PFAS accounted for varying proportions of EOF in the samples (Figure 2). The current study measured up to 64 targeted PFAS in the samples aiming to close the mass balance of EOF. The majority of the EOF were

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mainly explained by legacy PFAS such as PFOA, PFNA, PFDA, PFHxS, PFOS which was similar to the results of other studies.[32],[33],[34],[35] However, TFA stood out in the mass balance analysis; the median proportion of unknown fluorinated chemicals decreased from 62 to 29% (Figure 2) when TFA was excluded, as the concentrations of TFA exceeded other PFAS measured. It has been suggested in other studies that TFA might contribute to some proportions of the unrecognized EOF in the samples,[36],[37] and the current study further confirmed the importance of measuring TFA to reduce the proportion of unrecognized EOF in the mass balance analysis.

TFA in human blood

As shown in the results from targeted analysis, concentrations of TFA were almost 2 times higher than those of PFOS in Cohort 1 (EuroMix study) suggesting significant human exposure to TFA. Since no recovery correction was carried out, real concentrations could be even higher. On the other hand, the determination of TFA only relied on a single transition in the multiple reaction monitoring (MRM) that may pose the possibility of reporting false positives. To ensure our TFA identification is valid, a subset of samples was analyzed with additional LC-MS/MS using the method reported by Covaci et al.

Holaday (1977)[38] reported a half-life of TFA in human blood of approximately 16 hours, which is much shorter than other PFAS such as PFOS and PFOA. The detection of TFA in human samples at current concentrations may suggest exposure of significant amounts of TFA in daily life. TFA is known to be used in many industrial applications, consumer products and it is one of the final degradation products of chemicals used in cooling appliances potentially resulting in a persistent contamination of the environment and increasing human exposure.[39] A study[40] from Indiana, US showed that TFA was the only PFAA for which serum

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concentrations significantly correlated with both dust and water concentrations (Spearman correlation coefficients (r)=0.40, *p<0.001* and r=0.28, *p=0.01*, respectively). Recent studies[41],[42] reported TFA concentrations in drinking water in Sweden and Norway which ranged from 70 to 720 ng/L; specifically, the concentration in Oslo was 230 ng/L. These reported TFA concentrations in drinking water were below the guideline values for Germany (60 µg/L),[43] Denmark (9 µg/L) [44] and the Netherlands (2.2 µg/L).[45] The German environmental protection agency (UBA) proposed to classify TFA as toxic to reproduction based on a recent study from Bayer on the reproductive toxicity of TFA in rabbits, which found severe fetal malformations.[46] Further investigations are needed to understand human exposure to TFA and its impact on human health.

Previous studies also suggested that the unknown fraction may be caused by the metabolization of fluorinated pharmaceuticals carrying CF_3-groups in their molecular structure. A recent study confirmed that fluorinated pharmaceuticals accounted for up to 63% of the EOF, and their contribution increased in recent years.[47] We did not survey or measure any pharmaceuticals in the samples from Cohort 1 due to the lack of ethical clearance. However, interesting and important information can be obtained from the subjects in Cohort 2 that had taken fluoxetine, which is one of the CF_3-containing drugs used to treat depression, obsessive-compulsive disorder, some eating disorders, and panic attacks. Significant higher concentrations of EOF were observed in the subjects who had taken fluoxetine compared to the control group, even though for two subjects from the control group who had elevated concentrations of target PFAS contributing to the EOF (Figure 1). It is known that fluoxetine will be metabolized into fluoxetine glucuronide, norfluoxetine, seproxetine, norfluoxetine glucuronide and other metabolites.[48] In our current investigation, only seproxetine and fluoxetine were measured, thus part of the unrecognized EOF may be attributed to the transformation products of fluoxetine that we did not measure. Fluoxetine and norfluoxetine have long elimination half-lives, resulting in the drug remaining in the body for several weeks, even after discontinuation. The metabolism of fluoxetine is

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extensive, with approximately 2.5% of the administered dose excreted unchanged in the urine.[49] Therefore, consistent use of the prescribed drug or even after stopping the use might have resulted in elevated concentrations of EOF. Further, we have no information on other fluorinated pharmaceuticals consumed by the subjects in Cohort 2 donors, which could further explain the variation in FOF and TFA in our study.

Temporal variations of EOF

Several studies have conducted temporal analyses of EOF. However, these analyses were conducted at intervals of one year or longer and were also cross-sectional.[50],[51],[52] The current investigation is the first temporal analysis of EOF on a longitudinal basis (paired samples of the same individuals) and with a shorter interval between sampling occasions to also catch changes in fluorinated compounds with shorter half-lives such as TFA. As shown in Table 2, approximately 36% of the samples exhibited significant changes (25%), either an increase or decrease. The changes in EOF were not due to changes in legacy PFAS as these PFAS have long half-lives in humans[53] and our results also showed no significant changes (25%) of these PFAS in the subjects. One hypothesis for the observed changes in EOF may be exposure to fast-eliminating compounds like TFA, which can cause fluctuations due to its short half-life (16 hours)[54] and its significant contribution to the total PFAS. However, this assumption is based on the direct exposure to TFA, the parent compound, without taking into consideration the duration of transformation from unknown precursor compounds to TFA and some other unknown compounds. Nine individuals showed significant changes in TFA concentrations, but only 3 pairs showed significant changes in both EOF and TFA. Further characterization of unrecognized EOF present by using chemical oxidation may help interpret the results.

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Associations between the proportion of unknown fluorinated chemicals and dietary habits or use of personal care products

A previous study utilising the same samples identified several foods and personal care products as potential sources of exposure to certain targeted PFAS.[55] In the current study, we investigated whether or not there might be a specific factor (diet or use of personal care products (PCPs)) driving the proportion of unknown fluorinated chemicals in the samples. Inconsistent results were observed between the proportion of unknown PFAS and both dietary habits and personal care product use in T1 and T2 samples. As results shown above, no dietary habits nor use of personal care products were associated with the proportion of unknown fluorinated chemicals in samples from T1 and there was an association with consumption of Potato/vegetables in samples from T2. Several studies have reported varying proportions of unknown fluorinated chemicals in cosmetic products,[56],[57] as well as in fish[58] and shrimp.[59] Since it is not yet known how much unrecognized fluorinated chemicals may be present in food items or personal care and other consumer products, and the majority of these unrecognized fluorinated chemicals from which parts of the diet or specific PCPs; it is not surprising to obtain these results. As shown from results of Cohort 2, elevated levels of EOF and unrecognized EOF has been linked to the consumption of fluorinated pharmaceuticals, these sources may play an important role in EOF and unrecognized EOF.

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Applicability of EOF for biomonitoring

Results from a previous study showed EOF can be used as screening tool for PFAS exposure as the concentrations of EOF in the exposed group was almost one order magnitude higher than the control group.[60] Results from the current EuroMix study represented background exposed population, the results of the current study can serve as a baseline study before implementation of a future general PFAS restriction. In Cohort 2, two subjects in the group not taking fluoxetine had elevated concentrations of EOF, with high concentrations of 6:2 FTSA and PFHxS found in their blood samples. This finding further supports the use of EOF as a screening tool for PFAS contamination. Elevated concentrations of EOF were also observed in subjects taking fluoxetine, confirming the previous hypothesis that the consumption of fluorinated pharmaceuticals may increase EOF concentrations in the blood. Another study, however, reported similar EOF concentrations between subjects who reported using fluorinated pharmaceuticals and those who did not.[61] Therefore, in future studies it is important to obtain information from subjects about the types of medication taken before giving blood samples to avoid misinterpretation. Although some fluorinated pharmaceuticals are considered PFAS according to the newly adopted definition,[62] the purpose of using EOF in biomonitoring is to detect exposure to unrecognized PFAS, not known pharmaceuticals. Notably, around 36% of the paired samples showed more than 25% difference in EOF concentrations between the two samples collected 2 to 3 weeks apart, suggesting humans are exposed to fast eliminating unrecognized EOF in daily life.

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