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Abstract

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic chemicals present in the environment and defined as persistent organic pollutants (POPs). The interest in these forms of contaminants is related to the toxic consequences for health derived from exposures and bioaccumulation processes. The present research aims at assessing differences in the exposure of PFAS in the Italian population by hair analyses. To this aim, 20 compounds of the PFAS family were investigated in hair of 86 Italian subjects distributed across the regions of Veneto, Emilia-Romagna, Lombardy and Marche. The applied method was *ad hoc* developed in a previous research and included SPE extraction and LC-QTOF analysis. In the analyzed population, 66.4% had quantifiable amounts of one or more PFAS molecules (up to 4 compounds); mean PFAS content, expressed as sum of PFAS, was 0.1457 ng/g, ranging from “not detected” to 0.85 ng/g (SD 0.1867). PFOA and PFOS were the chemicals most frequently detected, with mean concentrations of 0.1402 ng/g and 0.1155 ng/g, respectively. PFBA was detected in 9.3% of subjects with a mean concentration of 0.3760 ng/g; PFNA in 3.5% of subjects with mean concentration 0.12 ng/g; PFDA was found in one subject at the concentration of 0.541 ng/g. PFUnA

and PFHxS were detected below the limit of quantification. The overall results displayed differences in the presence and prevalence of PFAS in hair of the Italian population on a geographical base. On the contrary, no significatively differences in the amount of PFAS were observed when considering gender or age classes. On this base, hair can be considered a good diagnostic tool to assess PFAS exposure on a regional-scaled base. Of course, more studies are required to infer PFAS internal dose from hair results due to its peculiar detection window and to interpretative issues derived from external contamination.

1 Introduction

The acronym "PFAS" stands for "per- and polyfluoroalkyl substances" (ITRC, 2020), a class of fluorinated compounds produced by anthropogenic activities. Most of them are already defined as persistent organic pollutants (POPs) by the Stockholm Convention. They are produced by industrial activities and can be found in surfactants, carpets, fire-retarding and food packaging. The most studied PFAS include the short chain PFOA (perfluorooctanoic acid) and PFOS (perfluorooctane sulfonic acid). A search performed on March 2021 on Pubmed database concerning the keywords "PFAS", "exposure" and "human" connected by Boolean operator "AND" retrieved more than 600 scientific papers published in English from 2001 to 2021, with a decisive positive trend starting from 2012. This date comes shortly after the scientific studies financed by DuPont, one of the major producers of PFOA, on the correlation between PFOA exposure and malignancies in mice. The interest in these forms of contaminants has then exponentially increased and the toxic

consequences deriving from exposures and bioaccumulation processes are still being gathered. It must be said that while the level of exposure appears to have declined, due to a ban in PFOA production, new studies demonstrate the presence of some of these chemicals also in the ecosystem of the developing countries (Baabish et al., 2021), signing a likely global distribution. Moreover, recent research stressed the aggravating point referred to the toxicity of early exposure in younger subjects, which is because PFAS are endocrine disruptors compounds (EDC) and may interfere with pubertal development and reproductive function at various levels (Marks et al., 2021). Also, epigenetic modifications have been suggested in relation to PFAS exposure both in adults, at birth (Kim et al., 2021) and on the fauna (Bernardini et al., 2021). On one hand, environmental burden, and the consequences of the presence of these contaminants have been known since the beginning of 2000s, and extensive biomonitoring studies throughout the world have been consequently implemented. These included mainly water basins (Italian Regional Environmental Agency, 2021; Morganti et al., 2021) and seafood (Glaser et al., 2021; Ali et al., 2021; Point et al., 2021). On the other hand, rising data on their toxicity directly affecting humans are posing increased issues on how to estimate total body burden and which matrix best represents this data. The results concerning the environment show that the degradation of these compounds is extremely slow, bioaccumulation occurs through the chain food and that novel PFAS are increasingly detected in European surface waters (Xiao, 2017). Regard to humans, PFAS exposure estimation is usually performed by serum analysis, even though the so far studied matrices include blood,

urine, breast milk and semen (Berg et al., 2021; Calafat et al., 2019; Zheng et al., 2021; Pan et al., 2019). Interestingly, hair, which is traditionally used for retrospective exposure studies to xenobiotics in clinical and forensic fields has been scarcely taken into consideration and only few publications are present in the literature. In fact, an analogue search performed with the terms “PFAS AND hair AND exposure” revealed 5 papers in the years 2015-2021 with no trend observed. Three papers proposed and applied new analytical methods for PFAS determination in hair (Piva et al, 2021; Kim, 2017; Alves et al., 2015), the remaining provided biomonitoring data (Li et al., 2021; Ruan et al., 2019, Wang et al., 2018). PFOS and PFOA were the analytes studied by all methods, and therefore frequently detected, while other PFAS varied according to specific analytical method. Data regarding PFOS and PFOA were in the range “not detected”-51.03 ng/g and 0.046-2.65 ng/g, respectively. PFHxA, PFHxS, PFHpA, PFDA, PFDS, PFNA, PFUnDA were also detected in biomonitoring studies. One disadvantage of using hair for biomonitoring purposes certainly rely on the high sensitivities required to detect these chemicals, in the order of few ng/g, reachable only by using high-performance instrumentation coupled to some degree of sample purification. In fact, concentrations in the hair samples are at least 1 order of magnitude lower respect to serum, which is at 1-10 ng/g. Also, interpretation issues (i.e. external contamination) may arise when considering hair data. However, the advantages of low sampling invasiveness, storage stability and little effect on daily fluctuation due to food consumption could be exploited in large-scale investigation across population. In fact, hair provide useful

information both on past exposure to xenobiotics and magnitude of bioaccumulation, already for some ubiquitous contaminants such as dioxins, polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Moreover, the relationships observed between the concentration of chemicals in hair and in other matrices (fluids and tissues) demonstrated that hair was representative of the internal dose and in some cases, the superiority of hair over serum analysis for identifying exposure was assessed (Appenzeller and Tsatsakis, 2012).

The present research aims at exploiting hair analyses to assess differences in the exposure of PFAS in the Italian population. To this aim, 20 compounds of the PFAS family were investigated in hair of 86 Italian subjects living in four Italian regions: Veneto, Lombardy, Emilia-Romagna and Marche (for geographical distribution see figure 1). Recently, the Veneto region was under study by the World Health Organization (WHO), who documented the story of PFAS contamination of the drinking water of 21 municipalities. Industrial activity in the area had polluted both surface waters and ground water, as well as the drinking water of approximately 127 000 citizens. Monitoring conducted by the authorities of the Veneto Region found PFOS in 63-100 % of the locations sampled and PFOA in 100 % of the sites. Moreover, a joint study with the Ministry of Environment, Land and Sea found that the Po River, which crosses Lombardy, Veneto and Emilia-Romagna regions, had the highest concentrations of PFOA of all the European rivers studied (WHO, 2007). In this frame, the idea of this study is to statistically compare the results in prevalence

and concentration of PFAS in hair of the inhabitants of the four Italian regions, with particular attention on the Veneto population. Lastly, a correlation of PFAS concentration with gender and age data was carried out.

2 Materials and methods

2.1 Materials

PFAS compounds (> 98% purity): potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate (11Cl-PF3OUdS); potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS); perfluoro-n-butanoic acid (PFBA); potassium perfluorobutanesulfonate (PFBS); perfluorodecanoic acid (PFDA); perfluorododecanoic acid (PFDoA); perfluoro(2-ethoxyethane)sulfonic acid (PFEESA); sodium perfluoroheptanesulfonate (PFHpS); perfluoroheptanoic acid (PFHpA); potassium perfluorohexanesulfonate (PFHxS); perfluorohexanoic acid (PFHxA); perfluoro-3-methoxypropanoic acid (PFMPA); perfluoro-4-methoxybutanoic acid (PFMBA); Perfluoro-n-nonanoic acid (PFNA); potassium perfluoro-1-octanesulfonate (PFOS); Perfluoro-n-octanoic acid (PFOA); Perfluoro-n-pentanoic acid (PFPeA); sodium perfluoro-1-pentanesulfonate (PFPeS); perfluoroundecanoic acid (PFUnA); 4,8-Dioxa-3H-perfluorononanoic acid (ADONA) were obtained from Wellington Laboratories (Guelph, Ontario, Canada). Mass-labelled (13C) perfluoro-n-[1,2-13C2] octanoic acid (M2PFOA) and mass-labelled (13C) sodium perfluoro-1-[1,2,3,4-13C4] octanesulfonate (MPFOS) at chemical purities > 98% and isotopic purities of > 99% were used as internal

standards (I.S.) and were also from Wellington Laboratories. API-TOF reference mass solution was from Agilent Technologies (Santa Clara, USA). Perfluoroalkyl compounds and mass-labelled analogues were diluted in methanol to working standard solution at concentration of 10 ng/ml. Solutions were stored at -20 °C and left at room temperature at least 2 h for equilibration prior use. Solvents like methanol and acetonitrile for mobile phases and purification steps (all LC–MS grade) were purchased from Merck (Darmstadt, Germany). Formic acid 98-100% for LC-MS was also from Merck. Water for mobile phase was obtained by Sartorius Arium mini apparatus (Sartorius, Goettingen, Germany). Ammonium acetate was acquired from Sigma-Aldrich (S. Louis, MO, USA). Bond Elut-ENV (200mg, 6 ml) cartridges for solid-phase extraction were from Agilent Technologies (Santa Clara, US).

2.2 Hair samples preparation

The hair samples were collected from the general population of four Italian regions (Veneto, Lombardy, Emilia-Romagna and Marche). The study was approved by the Ethical Committee of the University of Bologna and informed consent was obtained by the participants. All hair samples were collected from the vertex posterior region of volunteers. From these samples a proximal 3-5 cm segment was used. The procedure for sample treatment is described in a previously published study to which the reader is referred to, also for consultation of method validation data (Piva et al., 2021). Briefly, hair samples were decontaminated by subsequent rinses with water and acetone (two times each). To define solvents and times of washing, all solvents were analysed to check for absence of PFAS in solution during the early phases of

method development. Two subsequent rinsing with water and acetone showed suitable to obtain a final negative wash. This was important to guarantee that the outside of the hair was sufficiently washed to remove PFAS possibly due to product usage. Hair samples were left to dry at room temperature and then cut into small pieces. Analytes extraction from the matrix (100 mg) was performed by ultrasonication in acetonitrile. Sample clean-up was by solid phase extraction (SPE) by using Bond elut-ENV cartridges. Extracts were taken to dryness under a flow of nitrogen at 40°C using a metal heating block. Finally, samples were reconstituted in 500 µl water/methanol, 90/10 (v/v).

2.3 Analytical determination of PFAS

The LC MS system consisted of an Agilent 1290 Infinity II high pressure liquid-chromatography (HPLC) system coupled to an Agilent 6546 quadrupole- time-of-flight mass spectrometer (Q-TOF, Agilent Technologies, Santa Clara, CA). Separations were carried out in an EC-C18 column (2.1 x 100 mm, 1.9 µm), (Agilent Technologies, Santa Clara, CA), while a second LC C18 column (EclipsePlus -C18, 3.0 x 50 mm 1.8 µm) was placed after pump exit to delay any perfluorinated interferents originating from fluidic system. The mobile phase A consisted of a solution of 0.1 % formic acid/20 mM ammonium acetate in water (v/v) and mobile phase B of a solution of 0.1% formic acid in acetonitrile (v/v). Flow rate was 0.4 ml/min. The gradient was as follows: A-B, 97%-3% at time 0, A-B, 75%-25% at 1 min, gradient to A-B 15%-85% from 1 to 9 min, gradient to A-B 3%-97% from 9 to 10, isocratic A-B, 3%-97% for 2 min, equilibration at 3% B up to 15 minutes. The

injection volume was optimized and the final result was 20 µl. The Q-TOF instrument was operated in negative ion mode and source parameters were set as follow: capillary at 3500 V, gas temperature at 320°C, sheath gas temperature at 350 °C, drying gas at 8 l/min, nebulizer 35 psi, sheat gas flow at 12 l/min. All source parameters were optimized under LC conditions. Analytes were detected in high accurate mass scan in the range 100-1000 m/z at a rate of 2 spectra/sec and 3376 transients/spectrum. References masses were acquired throughout the run and were 112.9855 and 980.0163 m/z. Identification of each compound in matrix was by accurate mass (≤ 5 ppm) of the [M-H]- measurement, isotopic pattern distribution (isotope abundance and isotope spacing match) and retention time compared to standards. Tune parameters: 10 GHz, negative mode, m/z range 3200, high resolution.

Sensitivity of the method, expressed as limit of detection (LOD) were in the range of 0.02- 0.07 ng/g for PFBS, PFEESA, PFPeS, ADONA, PFOA, PFNA, PFHxS, PFHpS, PFOS, 9-Cl-PF3ONS, 11-Cl-PF3OUdS and 0.09-0.12 ng/g for PFBA, PFMPA, PFPeA, PFMBA, PFHxA, PFHpA, PFDA, PFUnA and PFDoA. Limit of quantification (LOQ) was in the range of 0,05-0,15 ng/g for PFPeA, PFBS, PFEESA, PFPeS, PFOA, PFNA, PFHxS, PFHpS, PFOS, 9-Cl-PF3ONS, 11-Cl-PF3OUdS and 0.2-0.5 ng/g for PFBA, PFMPA, PFMBA, PFHxA, PFHpA, ADONA, PFDA, PFUnA and PFDoA.

2.4 Statistical analysis

Descriptive statistics was obtained for the totality of hair samples, as well as considering each geographic area separately; epidemiological characteristics of subjects belonging to different geographical areas were compared by means of parametric and non-parametric one way and two-way ANOVA.

For each hair sample, the total amount of PFAS was calculated as a sum of the single substances. Normality tests (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov) were used to assess if the hair content of PFAS followed a Gaussian distribution. Since no normality test was passed, non-parametric statistics was applied to this variable. For statistical analysis, a 0 value was considered when the concentration of PFAS in the sample was below the limit of quantification (LOQ) in case of quantitative analysis (mean, SD), while for frequency calculation all detectable data (i.e. with values ≥LOD) were considered.

In the total sample, non-parametric t-tests and ANOVA were performed by grouping the test field (PFAS) for either age intervals (0-20, 21-40 and >40 years), gender or geographic areas, to check if the distribution of PFAS was the same across the tested categories (null hypothesis). Statistical analyses were performed by Prims (GraphPad 8.2.1) and SPSS (IBM SPSS Statistics 26.0.0.2) setting $p < 0.05$ for significance.

3 Results

In total 86 hair samples were collected and analyzed, mean age was 32 years, ranging from 18 to 65 (SD 11). Only 13 subjects (15%) had no detectable amounts of any PFAS, while 16 (18,6%) had amounts below LOQ and the remaining 66.4% had

quantifiable amounts of one or more PFAS molecules (up to 4 compounds). The most prevalent association was PFOA/PFOS (45%), followed by PFNA/PFOA (19%), PFOA/PFBA (8%) and PFDA/PFOS (6%). The mean sum of PFAS was 0.1457 ng/g, ranging from 0 to 0.85 (SD 0.1867). PFOA and PFOS were respectively detected at mean concentrations of 0.1402 ng/g and 0.1155 ng/g. PFBA and PFNA were detected at considerably high mean concentration of 0.3760 ng/g and 0.12 ng/g, respectively, PFDA was scarcely detected (Figure 2). More details are shown in Table 1. Mean age of subjects collected in Emilia-Romagna was 32 years; mean PFAS amount in hair was 0.0959 ng/g (SD 0.1699) with decreasing prevalence of PFOS, PFOA, PFBA, PFNA and PFDA. Mean age of subjects collected in the Veneto region was 33 years; mean PFAS amount was 0.2072 ng/g (SD 0.2051) with prevalence of PFOS, PFOA, PFBA, PFNA and PFDA. Mean age of subjects obtained from the Lombardy region was 33 years; mean PFAS hair concentration was 0.0986 ng/g (SD 0.0963). The frequency of PFOA was the highest at 70%, followed by PFOS, PFNA, PFDA. Lastly, mean age of subject obtained from the region of Marche was 30 years; mean PFAS content was 0.1599 ng/g (SD 0.1249). PFOA and PFOS were both detected at high frequency among the analyzed population, followed by PFNA, PFUnA and PFHxS. Details of descriptive statistics of the sum of PFAS for each geographical area is presented in Table 2 and depicted in figure 3 and figure 4. As can be noted, Emilia-Romagna and Veneto shared the same distribution of PFAS, while the Marche region displayed a totally different distribution respect the others, with occurrence of PFHxS and PFUnA. Lombardy was the only region with the highest occurrence of PFOA. In consideration

of the homogeneous epidemiological characteristics across the population of the different geographical areas, a statistical evaluation of the difference in the total amount of PFAS in hair was considered among regions (hypothesis 1). Moreover, the possibility of a statistical difference between gender (hypothesis 2) and age (hypothesis 3) in the total PFAS concentration in hair was evaluated. By testing hypothesis 1, a statistically significant difference was found based on geographical areas only between the areas of Veneto and Emilia-Romagna. By testing hypothesis 2 and 3, no statistical difference was observed according to statistics (Figure 5 and figure 6).

Members of the same family group (in total 5 families) were included in the present study in search of possible overlapping trend, in consideration of the bioaccumulation circumstances through environment and nutrition. The results confirmed the same types of chemicals for members of the same family in most cases, but not strictly observed in all cases, indicating a possible subjective component in bioaccumulation.

Discussion

The herein study on retrospective human exposure to PFAS, by comparing 86 hair samples is the widest so far proposed. A very recent research conducted in Hong Kong considered hair from 53 subjects at preschool age (Li et al., 2021), while the first nationwide survey in India considered 39 samples from 14 cities (Ruan et al., 2019). In both studies the aim was to identify the predominant PFAS in the population with special attention on statistical significance for subject characteristics or regional distribution. In the former study, seven PFAS were evaluated in hair

samples from children at age 4-6 years old. PFOS and PFDDA were not detected in the hair, while PFOA was the major chemical found in hair with a detection frequency of 70%. PFHxA and PFHpA were also detected. Mean concentrations were in the range of 0.003-0.248 ng/g. Finally, a statistical difference in the sum of PFAS was observed in hair between girls and boys. In the latter, 25 PFAS were monitored, 9 of them were found in the population and the most predominant PFAS were PFOA and PFHxS (frequency > 50%), followed by PFOS and PFDS, then PFBS, PFBA and N-EtFOSA. Also, PFHxA and 6:2 FTUCA were found. Mean concentrations were in the range of 0.08-0.8 ng/g, highest concentrations for PFOS and PFHxS. The study confirmed regionality as a factor influencing PFAS hair content. A different study performed in China by Wang and colleagues (Wang et al., 2018) analysed 6 PFAS in 4 different matrices of 39 subjects. PFOS and PFOA were the most frequently detected compounds in hair, 92% and 72% respectively, at 0.6 and 0.25 ng/g. We performed the research of 20 PFAS in the hair of 86 subjects. Our findings were mostly in agreement with the abovementioned studies, confirming PFOS and PFOA were the most frequently chemicals found in hair. However, mean concentrations for both analytes were generally lower when compared to data from China, India and Hong Kong (the most recent studies available in the literature). On one hand this demonstrates a worldwide diffusion of these compounds also in hair, as observed for other biological matrices (blood, serum). On the other hand, PFOS and PFOA concentrations widely differ on a regional base as well as detection frequency of other PFAS. For this reason, a worldwide comparison of the whole data is more

difficult. The occurrence of other PFAS may be more related to regional factors concerning nutrition, air and water pollution. In fact, contrary to other studies, PFHxA, PFHpA, PFBS were never detected in our samples and only a very low frequency of detection for PFHxS was found. PFBA, PFNA and PFDA were instead detected in our study at concentrations equivalent (PFBA) or greater (PFNA, PFDA) than that reported in the mentioned studies. Also in our data, regional differences were observed. In particular, data from the Marche region showed a generally different occurrence of PFAS when compared to other regions (figure 4), with the presence of PFHxS and PFUnA, not detected anywhere else. It should be clarified that subjects submitted to the study were predominantly living in the city of Ancona, on the coast of the Adriatic Sea, differently from the population collected in Veneto, Emilia-Romagna and Lombardy, which was sampled in cities far from the sea. A different PFAS load due to a major seafood consumption or to aerosol pollution from sea could be one of the possible explanations since some data already suggest an association between seafood consumption and PFAS presence (Dartey et al., 2021; Bjorke-Monsen et al., 2020; Thépaut et al. 2021). Even though other chemicals in the Adriatic Sea are not as high as in other basins, high levels of PFOA, PFOS and PFHxS were found in 2013 (Loos, 2013). However, in this case, the observed demographic patterns might not directly reflect differences in exposure sources but could also be ascribed, in the absence of other data, to dissimilarities in adsorption and metabolism. Data concerning the higher mean concentration of PFAS in hair of the Veneto population is instead supported by the finding on drinking water. A study

financed by the European Commission found values exceeding by a factor of 130 for PFOS and 66 for PFOA the imposed limit of 0.1 μg/L for each individual PFAS in waters sampled in municipalities of the Veneto region (European Environment Agency, 2021). The correlation between PFAS presence in biological matrices and phenotypical gender is controversial in literature. Although some studies found a difference in the total burden of PFAS between females and males (Li et al., 2021; Ingelido et al. 2020), with males accumulating more PFAS, others, did not (Ruan et al. 2019; Wang et al. 2018). Neither our study did reveal any statistical difference between the analysed PFAS content between the two genders. In the perspective of new studies, the gender factor needs surely to be taken in consideration more deeply, since at the moment a clear tendence on hair is lacking (Jian et al., 2018). Also, data analysed through age classes (<20; 20-40; >40 years old) missed to reveal any statistical difference, confirming the vertical distribution of these chemicals through the population. This could be explained on the fact that PFAS exposure already begins at the early stage of development in utero and continues with breastfeeding (Goeden et al., 2019; Macheka et al., 2021). Then, due to the PFAS peculiar pharmacokinetics and pharmacodynamics properties, even short exposures during infancy have dramatic impacts on serum levels for many years.

Finally, as a general reflection of this research, which should be intended as a preliminary study on the prevalence and abundance of PFAS in hair of Italian population, some limits exist and should be considered. Major limits are represented by the total number of samples that, although larger than the studies present at the

moment, hamper a perspective on the entire Italian population. Data on youngers is of course limited by the number of samples at early ages, but results are consistent with the consulted literature. At this stage, a correlation with serum concentrations, considered the gold standard measurement for PFAS body burden, is missing. This would be of great importance also to understand the biological disposition of these xenobiotics in human matrices and to find appropriate interpretative limits. As future perspective, the use of high (resolution) accurate mass measurement will be exploited for the untargeted mass-spectral detection of new perfluorinated analytes (such as chloroperfluoropolyether species) in hair of subjects living in contaminated areas (Washington et al., 2020).

4 Conclusions

The aim of the study was to analyse hair sampled from the population of four Italian regions and to evaluate PFAS presence. The research revealed a difference in the presence of PFAS in hair of the Italian population according to geographical distribution. A significative difference in PFAS concentration was found between population living in Veneto and Emilia Romagna regions. These data confirm that hair reflect differences in the amount and type of incorporated PFAS on a geographical-based distribution, also for neighbouring areas. This support the fact that hair could be used as a complementary matrix for assessing PFAS exposure, as verified for other chemicals. The advantages of low sampling invasiveness and storage stability could be exploited in large-scale investigation and when sample invasiveness is hampered (i.e., neonates). Of course, more studies are required to

understand the difference between hair PFAS baseline levels and levels indicating medium/high exposition and for example to estimate the total body burden of PFAS on the base of hair content. However, as concluding remarks, data on PFAS pharmacodynamics and pharmacokinetic in human should not be neglected to fully understand the biological disposition of these xenobiotics in matrices.

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