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Biomonitoring of perfluorinated compounds in adults exposed to contaminated drinking water in the Veneto Region, Italy

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ABSTRACT

In 2013 a contamination of drinking water by perfluoroalkylated substances (PFASs) was discovered in areas of the Veneto Region (northern Italy). In this study the exposure to PFASs of people living in the aforesaid areas was characterized: contaminant serum concentrations were measured and compared with those of a control population group living in neighboring areas at background exposure (based on available drinking water data). The enrolled population was also genotyped for the OATP1A2*3 allelic variant, possibly affecting PFAS excretion and hence the internal dose.

The difference in PFAS concentrations between exposed and not exposed subjects was significantly larger for nine of the 12 substances analyzed, and confirmed that water contamination had resulted in an appreciable high exposure of the residing population over time.

Within the group of exposed subjects, subgroups at different exposure levels were identified. The contamination of drinking water of the residence area was found to be the main factor influencing PFAS serum levels; in addition to water contamination, other relevant influencing factors were sex, the years of residence and raising own livestock. No relationship with the genetic trait for the studied renal transporter was evidenced.

These results provide a baseline characterization of PFAS exposure of the monitored population groups for further studies, planned to be carried out in the near future.

1. Introduction

Per- and polyfluorinated alkyl substances (PFASs) are used in a wide range of industrial applications and commercial products (e.g. paper coatings, insecticides, paints, fire-fighting foams, protective coatings for fabrics and carpets) due to their unique chemical-physical properties, in particular the capability to repel oil, grease and water. The strength of the fluorine-carbon bonds confers to PFASs a high thermal and chemical stability, and resistance to biodegradation in the environment and in the human body.

As a function of the carbon chain length, several PFASs are subject to long-range transport, and are capable to bioaccumulate along the food webs. So far, PFASs have been detected in soil, air, water bodies, sediments, dust, and biota including humans (ATSDR, 2015; Danish Environmental Protection Agency, 2015).

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) represent the most widely studied members of PFAS, because of their extensive use and persistence, and consequent presence in the environment and in the human body (ATSDR, 2015; Danish Environmental Protection Agency, 2015).

PFASs show a good mobility in water environments due to their amphiphilic nature. PFAS bioaccumulation potential is not related to the partition coefficient between plasma and the body lipid fraction (including the adipose tissue), but rather to chemicals' kinetic behavior, depending on i) rapid and extensive oral and inhalation absorption (> 90% in 0.25–1.5 h), ii) substantial binding to both plasma and liver proteins (mainly - but not exclusively - albumin) which represent the bioaccumulation reservoir, iii) lack of biotransformation, and iv) very slow urinary excretion with renal resorption (ATSDR, 2015).

The latter is particularly relevant since can markedly influence internal dose of the chemical and is a key step resulting in the marked species and gender difference in PFAS half-life. As an example, the elimination rate for PFOA in female rats is approximately 45 times faster than in males, 150 times faster than in non-human primates, and

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approximately 5000–9000 times faster than in humans (range: 1.5–14.7 years; average 3.8 years) (ATSDR, 2015; US EPA, 2016a). PFASs are indeed good substrates of organic anion transporters (OATs) and organic anion-transporting polypeptides (OATPs) in the luminal and basolateral membranes of renal tubular epithelial cells, which are responsible for active transport in both directions (i.e. secretion and resorption) (Andersen et al., 2008; Harada et al., 2005). The transporters are under hormonal control, show differences in expression levels and activity between species and genders and are also polymorphic, possibly resulting in interindividual difference in the elimination rate.

Humans are exposed to PFASs mainly by ingestion of food, including drinking water (EFSA, 2012; Hu et al., 2016; Hurley et al., 2016) and there were several cases of high level exposure due to contamination of drinking water worldwide (Emmett et al., 2006; Hoffman et al., 2011; Hölzer et al., 2008; Steenland et al., 2009; Vieira et al., 2008).

Many studies have been published on the adverse effects of PFASs, mainly PFOS and PFOA, in experimental animals; some epidemiological studies have also been carried out, especially in those areas showing a high level of contamination. Although the majority of available epidemiological studies is cross-sectional, and therefore does not allow to establish causality, in most investigations a positive association of PFOA with increased total cholesterol and low and high density lipoproteins in blood was observed, this suggesting dysfunction in lipid metabolism (Health Council of the Netherlands, 2013; ATSDR, 2015). Other positive associations with elevated uric acid serum levels, thyroid metabolism, and immunological endpoints were consistently reported; in addition, adverse health effects, including developmental effects in fetuses during pregnancy or in breastfed infants (e.g. low birth weight, neurodevelopment and reproductive impairment, precocious puberty) or cancer in occupationally exposed individuals have been reported; however, the effects were not consistently found across studies (ATSDR, 2015; Bundesgesundheitsbl, 2016; US EPA, 2016a; US EPA, 2016b; Health Council of the Netherlands, 2013) and the presence of confounders due to kinetics were also reported (kinetics could positively or negatively influence the outcome of the association as evidenced in some epidemiologic studies) (Verner et al., 2015; Wu et al., 2015).

As a consequence of their toxicity and persistence in the environment and the human body, PFOS has been included in the Stockholm Convention on POPs (Secretariat of the Stockholm Convention, 2009), while PFOA and perfluorohexane sulfonate (PFHxS) are under evaluation for inclusion (Secretariat of the Stockholm Convention, 2016 and 2017).

The PFAS water contamination that gave rise to this study occurred in the Veneto Region (north-eastern Italy) and was identified from the results of European and Italian research projects. In 2006 the European Project PERFORCE concluded an investigation aimed at performing an exposure assessment of perfluorinated substances in the European environment; the environmental matrices selected for monitoring included waters from European rivers. The river Po (northern Italy) showed the highest concentrations of PFOA (de Voogt et al., 2006). This finding was further investigated by extending the analysis of PFASs to other Italian rivers (Polesello et al., 2013): some sectors of the river Brenta basin (Veneto Region) were identified as “hot spots” for PFAS contamination, and PFASs were measured in both surface and ground water. Contamination involved also drinking water: nine perfluorocarboxylic acids (perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA) and perfluorodeconaoic acid (PFDoA)) and three perfluorosulfonates (perfluorobutane sulfonate (PFBS), PFHxS, and PFOS) were detected.

The contamination originated mainly from industrial emissions: a chemical plant located in the area has produced PFASs since 1968 (WHO, 2017). Due to PFAS presence in drinking water, the Veneto Region implemented a series of risk reduction measures to limit exposure of residents in the areas where water contamination was detected. Starting from 2014, the Region asked the Italian National Institute for Health (Istituto Superiore di Sanità, ISS) to provide scientific advice and support to evaluate population exposure to PFASs and the related health risk, also through designing and carrying out a human biomonitoring study. The study aimed at characterizing exposure to PFASs in people living in areas with evidence of drinking water contamination, compared to the control population group living in neighboring areas of the Veneto Region unaffected by water contamination. In addition, with the aim of identifying a potential genetic trait able to modulate internal exposure, for the first time, as far as the authors know, the enrolled population was genotyped for a polymorphic allele coding for OATP1A2. The rat transporter Oatp1a1 (Slco1a1 or Solute carrier organic anion 1a1) corresponding to the human OATP1A2, whose SNP (Single Nucleotide Polymorphism) has been studied here, is expressed on the apical membrane of the proximal tubule cells, where it has been shown to transport PFOA from the urine back into the proximal tubule cells facilitating renal reabsorption (Weaver et al., 2010). Although in vitro, perfluorocarboxylates were indicated not to be substrates for OATP1A2 (Yang et al., 2010), it was checked here in vivo whether the presence of at least one mutated allele could affect the body burden, at comparable level of external exposure.

2. Materials and methods

2.1. Study design

The study involved 507 subjects, 257 residing in municipalities in the areas under impact (Altavilla, Brendola, Creazzo, Lonigo, Montecchio Maggiore, Sarego, Sovizzo; identified with “E” for “Exposed” in the paper), and 250 residing in municipalities in areas at presumed background exposure (Dueville, Carmignano, Fontaniva, Loreggia, Mozzecane, Resana, Treviso; identified with “NE” for “Not Exposed”). The two areas were defined on the basis of the data available on PFAS contamination of the water supply system before the implementation of risk reduction measures. In each area participants were selected and stratified by gender and age (age classes: 20–29, 30–39, and 40–51 years). Each subject had resided in an area for at least 10 years.

The areas included in the study were under the territorial competence of different local health units (ULSS, Unità Locale Socio Sanitaria) of Veneto Region. Areas where contamination had been detected were under the jurisdiction of ULSS 5 and ULSS 6. Contamination of the two ULSS territories had affected both ground and well water but, depending on the structure of water distribution system, drinking water contamination had been detected nearly exclusively in ULSS 5. Areas not affected by water contamination (“background” contamination areas) were under the jurisdiction of ULSS 8, 9, 15, 22 as well as that part of ULSS 6 territory where absence of contamination had been demonstrated.

Sampling (5 mL of blood for chemical analysis, and 1 mL of blood for genetic analysis) was performed by ULSS laboratories between July 2015 and April 2016. A questionnaire was administered to participants to obtain information on anthropometric and socio-demographic characteristics, lifestyle, drinking-water consumption, and diet. The study was approved by the local ethical committees and each participant signed an informed consent before blood withdrawal. Not all the individuals enrolled for the chemical analysis gave his/her consent for genetic analysis (N = 217 and 241 for E and NE groups, respectively).

For chemical analysis, blood samples were centrifuged and the serum samples obtained were delivered to the ISS laboratory.

2.2. Chemical analysis

Serum samples were analyzed for nine perfluorocarboxylic acids (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, and

PFDoA) and three perfluorosulfonates (PFBS, PFHxS, and PFOS).

About 250 μL of serum were spiked with labelled internal standards. Extraction was performed in a centrifuge tube with acetonitrile (Sigma–Aldrich Corp, Saint Louis, MO, USA). After centrifugation the volume of the acetonitrile phase was reduced and transferred to an autosampler vial to undergo instrumental analysis (Ingelido et al., 2010). Instrumental analysis was carried out by HPLC (Waters Alliance 2695, Waters Corporation, Milford, MA, USA) interfaced with a triple quadrupole mass spectrometer (Micromass QuattromicroTM API, Waters Corporation, Milford, MA, USA) operated in the electrospray negative mode (De Felip et al., 2015).

Analyses were carried out by the Laboratory of Toxicological Chemistry of the ISS. The laboratory is accredited for the analysis of POPs according to ISO/IEC 17025. Since 2011, it has participated three times a year in the intercomparison exercise "AMAP Ring Test for Persistent Organic Pollutants in Human Serum" organized by the Institut National de Santé Publique du Québec, Centre de Toxicologie du Québec (Canada), and has always met the performance acceptability criteria for PFASs.

2.3. Genotype analysis

Genomic DNA extraction from whole blood was obtained using the QIAamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer's instructions.

Genotyping of rs11568563, a SNP present in OATP1A2/SLCO1A2 gene, was performed by TaqMan® SNP Genotyping Assay code C_25605897_10 (Thermo Fisher Scientific, Waltham, MA USA) as recommended by the manufacturer on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA USA). Polymorphic alleles of OATP1A2 are known to be functional determining a change in the activity toward different substrates and validated methods are readily available for their detections in the population. No checks were carried out for other variants since the number of enrolled individuals was not high enough to give statistical power to the study, considering the frequency of the mutated allele.

Known genotypes were included in each reaction as quality controls. Genotyping was performed on individuals of the entire study population by personnel blinded to NE and E exposed status. To validate genotyping procedures 5% of randomly selected samples were typed twice; all mutant homozygotes were confirmed by triplicate independent experiments (i.e. runs carried out on different days). The replicates were 100% concordant.

The characteristics of the studied polymorphism (SNP) are reported in Table 1.

2.4. Statistical analysis

The Shapiro-Wilk W test for normality was used to check the distributions of the variables; as none was normal, and almost all of log-transformed distributions did not satisfy normality (only PFOS data distribution was log-normal), non-parametric statistics was applied. In data processing, data below the limit of quantification (LOQ) were included as LOQ / √2. Mann-Whitney and Kruskal-Wallis tests were used to assess differences between groups in the concentrations of serum contaminants. Spearman's rank correlation coefficients were calculated to evaluate the correlations between the concentrations of each contaminant and the variables gathered through questionnaires. Analyses were carried out using Statistica 8.0 (StatSoft Inc. Tulsa, OK, USA).

The χ^2 test was applied to investigate if observed and expected genotype frequencies of OATP1A2/SLCO1A2 locus were in Hardy-Weinberg equilibrium. A $p < 0.05$ was considered to be statistically significant. Kruskal-Wallis and Mann-Whitney tests were performed to determine any differences in PFAS blood levels of individuals with three different genotypes: wild type (w/w; homozygotes) and mutated SLCO1A1 alleles (w/m and m/m; heterozygotes and homozygotes, respectively).

Table 1
SLCO1A2 genetic polymorphism (SLCO1A2*3).

Exon	Position	Base pair change	aa position	aa change	dbSNP IP	In vitro function[a]
5	516	A > C	172	E > D	rs11568563	rs11568563

aa = amino acid; dbSNP = SNP database.
[a] Gong and Kim, 2013.

Table 2
Characteristics of study participants.

	Exposed	Not exposed
	n (%)	*n* (%)
Gender		
Females	130 (51)	123 (49)
Males	127 (49)	127 (51)
Age (years)		
20–29	80 (31)	79 (32)
30–39	85 (33)	82 (33)
40–51	92 (36)	89 (35)
Body Mass Index (kg/m^2)		
Underweight	12 (5)	10 (4)
Normal	169 (66)	153 (61)
Overweight	60 (23)	68 (27)
Obese	16 (6)	19 (8)
Residence area		
Urban	128 (50)	78 (31)
Suburban	70 (27)	131 (52)
Rural	58 (23)	41 (16)
Grow own fruits/vegetables		
Yes	103 (40)	117 (47)
No	151 (60)	132 (53)
Raise own livestock		
Yes	21 (8)	55 (22)
No	233 (91)	192 (77)
Drink tap water		
Yes	142 (55)	92 (37)
No	115 (45)	158 (63)
Drink well water		
Yes	6 (2)	87 (35)
No	251 (98)	163 (65)
Use of water to cook[a]		
Tap water	249 (97)	142 (57)
Well water	4 (2)	108 (43)
Bottled water	10 (4)	3 (1)
Use of water to water fruits/vegetables		
Tap water	61 (24)	21 (8)
Well water	27 (10)	93 (37)
Use of water to water livestock		
Tap water	12 (5)	10 (4)
Well water	7(3)	46 (18)

[a] In this category the sum of the percentages is > 100 because 10 subjects used more than one type of water to cook.

3. Results and discussion

3.1. PFAS serum concentrations

The characteristics of study participants (sex, age, body mass index (BMI), residence area) together with information on lifestyle in relation to water use and consumption are shown in Table 2.

E and NE subjects were well matched regarding sex, age and BMI.

Table 3
Descriptive statistics of PFAS concentrations (ng/g) assessed in exposed and not exposed subjects.

Exposed	*N*	Minimum	P_5	P_{25}	Median	P_{75}	P_{95}	Maximum	< LOQ (%)
PFBA	257	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.58	3.59	93
PFPeA	257	< LOQ	< LOQ	< LOQ	< LOQ	0.10	0.31	0.46	74
PFHxA	257	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.27	0.68	80
PFHpA	257	< LOQ	< LOQ	< LOQ	< LOQ	0.10	0.21	0.42	49
PFOA	257	0.70	2.21	4.89	13.77	87.27	247.94	754.50	0.0
PFNA	257	< LOQ	< LOQ	0.40	0.61	0.88	1.54	2.46	5.8
PFDA	257	< LOQ	< LOQ	0.22	0.33	0.51	0.86	1.96	12
PFUdA	257	< LOQ	< LOQ	0.09	0.16	0.30	0.56	1.02	28
PFDoA	257	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.74	1.33	84
PFBS	257	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.69	4.26	69
PFHxS	257	< LOQ	< LOQ	1.22	2.98	6.92	21.20	43.43	8.9
PFOS	257	2.72	2.72	5.53	8.69	14.96	29.42	70.27	0.0

Not exposed	*N*	Minimum	P_5	P_{25}	Median	P_{75}	P_{95}	Maximum	< LOQ (%)
PFBA	250	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.16	0.35	83
PFPeA	250	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.13	0.22	60
PFHxA	250	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.09	0.26	82
PFHpA	250	< LOQ	< LOQ	< LOQ	< LOQ	0.07	0.13	0.26	34
PFOA	250	0.01	0.57	1.10	1.64	2.22	3.92	27.88	0.0
PFNA	250	0.03	0.23	0.41	0.58	0.80	1.34	7.72	0.0
PFDA	250	< LOQ	0.12	0.24	0.32	0.49	0.97	3.07	2.8
PFUdA	250	< LOQ	< LOQ	0.11	0.18	0.30	0.62	1.35	13
PFDoA	250	< LOQ	< LOQ	< LOQ	0.04	0.08	0.18	1.67	32
PFBS	250	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.11	0.36	82
PFHxS	250	< LOQ	0.18	1.42	2.49	3.99	6.04	9.14	4.8
PFOS	250	0.01	2.07	3.89	5.84	9.31	20.63	118.58	0.0

Major differences in lifestyle between the two groups concerned the residence area and the use of tap or well water. NE subjects used well water to drink, cook, and water vegetables more than E subjects (only 2% of E subjects declared to drink well water).

In all samples, PFOA and PFOS were above their respective LOQ. PFHpA, PFNA, PFDA, PFUdA, and PFHxS were above their LOQs in > 50% of the samples. The other five PFASs were less frequently detected: PFBA in 60 subjects (12%), PFPeA in 166 subjects (33%), PFHxA in 96 subjects (19%), PFDoA in 211 subjects (42%), and PFBS in 125 subjects (25%). Descriptive statistics (Table 3) and results of the Mann-Whitney test between E and NE subjects (Table 4) are reported for all the 12 PFASs analyzed; all the other statistical analyses were applied only to the seven PFASs determined in more than half of the samples.

Median concentrations of all substances were higher in E subjects with the only exception of PFUdA that was slightly higher in NE subjects. The largest difference was observed for PFOA: median value of the E group (13.77 ng/g) was eight times higher than the median value of the NE group (1.64 ng/g). PFOA is also the analyte that presented the larger data dispersion in E subjects with the P_{95} value 18 times higher than the median, while in NE subjects P_{95} was about twice the median.

Overall, a very strong correlation (Table 5) was observed among different analytes both in E and NE subjects. The significant positive correlation between PFAS may be due to the presence of a dominant common source of exposure in the area.

The difference in contaminant concentration between E and NE subjects (Table 4) was significantly larger for nine of the analyzed substances (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFDoA, PFBS, PFHxS, and PFOS). In order to identify subgroups of E subjects at possible incremental exposure, the Mann-Whitney test was also performed stratifying the E group by ULSS. Serum concentrations of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDoA, PFBS, PFHxS, and PFOS in subjects from ULSS 5 were significantly higher than concentrations observed in subjects from ULSS 6 and in NE subjects (Table 4). Subjects from ULSS 5 had the highest levels of PFOA, with a median value (74.21 ng/g) 45 times higher than that estimated for the NE group. Exposed participants from ULSS 6 appear to have serum concentrations of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFDoA and PFBS significantly higher than the NE participants.

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Table 4
Median PFAS serum concentrations (ng/g) in subgroups of subjects and results (p values) of the Mann-Whitney test. Significant p values ($p < 0.05$) are shown in bold.

	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFBS	PFHxS	PFOS
Exposed	0.12	0.07	0.05	0.05	13.77	0.61	0.33	0.16	0.12	0.15	2.98	8.69
Not exposed	0.03	0.03	0.02	0.03	1.64	0.58	0.32	0.18	0.04	0.04	2.49	5.84
p	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.33	0.87[a]	0.38[a]	**< 0.001**	**< 0.001**	**0.0022**	**< 0.001**
Exposed from ULSS 5	0.17	0.07	0.06	0.07	74.21	0.65	0.36	0.16	0.13	0.17	6.52	12.00
Not exposed	0.03	0.03	0.02	0.03	1.64	0.58	0.32	0.18	0.04	0.04	2.49	5.84
p	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.040**	0.85	0.76[a]	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Exposed from ULSS 6	0.04	0.03	0.03	0.04	4.62	0.60	0.32	0.16	0.09	0.09	1.69	6.32
Not exposed	0.03	0.03	0.02	0.03	1.64	0.58	0.32	0.18	0.04	0.04	2.49	5.84
p	**< 0.001**	**< 0.001**	**< 0.001**	**0.050**	**< 0.001**	0.55[a]	0.62[a]	0.24[a]	**< 0.001**	**< 0.001**	**< 0.001**[a]	0.26
Exposed from ULSS 5	0.17	0.07	0.06	0.07	74.21	0.65	0.36	0.16	0.13	0.17	6.52	12.00
Exposed from ULSS 6	0.04	0.03	0.03	0.04	4.62	0.60	0.32	0.16	0.09	0.09	1.69	6.32
p	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.039**	0.69	0.25	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

[a] Serum concentrations are higher in the not exposed group.

Table 5
Correlation between PFAS serum concentrations (ng/g) in exposed and not exposed subjects. Significant p values ($p < 0.05$) are shown in bold.

Spearman correlation	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFHxS	PFOS
	R (p)[a]	R (p)	R (p)	R (p)	R (p)	R (p)	R (p)
PFHpA							
Exposed		**0.418 (< 0.001)**	**0.190 (0.002)**	**0.179 (0.004)**	**0.212 (< 0.001)**	**0.298 (< 0.001)**	**0.334 (< 0.001)**
Not exposed		**0.182 (0.004)**	**0.134 (0.003)**	**0.141 (0.025)**	**0.124 (0.050)**	0.094 (0.136)	0.118 (0.063)
PFOA							
Exposed	**0.418 (< 0.001)**		**0.360 (< 0.001)**	**0.213 (< 0.001)**	**0.136 (0.029)**	**0.821 (< 0.001)**	**0.743 (< 0.001)**
Not exposed	**0.182 (0.004)**		**0.584 (< 0.001)**	**0.305 (< 0.001)**	**0.259 (< 0.001)**	**0.381 (< 0.001)**	**0.619 (< 0.001)**
PFNA							
Exposed	**0.190 (0.002)**	**0.360 (< 0.001)**		**0.467 (< 0.001)**	**0.342 (< 0.001)**	**0.406 (< 0.001)**	**0.550 (< 0.001)**
Not exposed	**0.134 (0.003)**	**0.584 (< 0.001)**		**0.687 (< 0.001)**	**0.529 (< 0.001)**	**0.226 (< 0.001)**	**0.706 (< 0.001)**
PFDA							
Exposed	**0.179 (0.004)**	**0.213 (< 0.001)**	**0.467 (< 0.001)**		**0.350 (< 0.001)**	**0.229 (< 0.001)**	**0.465 (< 0.001)**
Not exposed	**0.141 (0.025)**	**0.305 (< 0.001)**	**0.687 (< 0.001)**		**0.604 (< 0.001)**	**0.211 (< 0.001)**	**0.621 (< 0.001)**
PFUdA							
Exposed	**0.212 (< 0.001)**	**0.136 (0.029)**	**0.342 (< 0.001)**	**0.350 (< 0.001)**		**0.178 (0.004)**	**0.245 (< 0.001)**
Not exposed	**0.124 (0.050)**	**0.259 (< 0.001)**	**0.529 (< 0.001)**	**0.604 (< 0.001)**		**0.167 (0.008)**	**0.457 (< 0.001)**
PFHxS							
Exposed	**0.298 (< 0.001)**	**0.821 (< 0.001)**	**0.406 (< 0.001)**	**0.229 (< 0.001)**	**0.178 (0.004)**		**0.772 (< 0.001)**
Not exposed	0.094 (0.136)	**0.381 (< 0.001)**	**0.226 (< 0.001)**	**0.211 (< 0.001)**	**0.167 (0.008)**		**0.425 (< 0.001)**
PFOS							
Exposed	**0.334 (< 0.001)**	**0.743 (< 0.001)**	**0.550 (< 0.001)**	**0.465 (< 0.001)**	**0.245 (< 0.001)**	**0.772 (< 0.001)**	
Not exposed	0.118 (0.063)	**0.619 (< 0.001)**	**0.706 (< 0.001)**	**0.621 (< 0.001)**	**0.457 (< 0.001)**	**0.425 (< 0.001)**	

[a] Spearman's R correlation coefficient (p value).

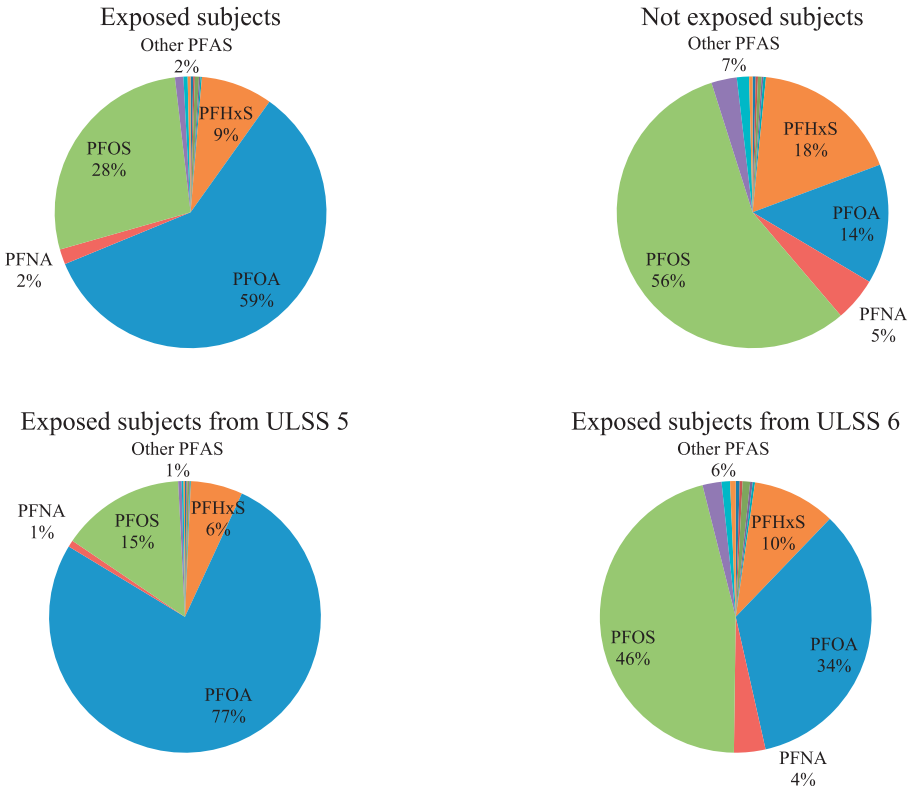

Fig. 1. PFAS distribution in human serum of exposed and not exposed subjects and exposed subjects from ULSS 5 and 6.

the different PFASs was observed for E and NE subjects (Fig. 1), For NE subjects, the greatest relative contribution derives from PFOS, as generally observed in studies involving subjects from the general population, whereas the contribution of PFOA is dominant in the E group, in particular in the ULSS 5 territory where the contribution to exposure from contaminated water was higher. In the ULSS 6 territory we observed an intermediate situation, as expected on the basis of information on water contamination.

3.2. Correlation between PFAS serum levels and gender, age, and BMI

Results of Spearman correlation and Mann-Whitney and Kruskal-Wallis tests are reported in Table 6.

Males showed serum concentrations significantly higher than

Table 6
Characteristics of study participants and relationship with PFAS serum concentrations in exposed and not exposed subjects. Significant p values ($p < 0.05$) are shown in bold.

Spearman correlation	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFHxS	PFOS
	R (p)[a]	R (p)	R (p)	R (p)	R (p)	R (p)	R (p)
Age (years)							
Exposed	**0.189 (0.002)**	0.013 (0.836)	0.103 (0.101)	0.116 (0.063)	0.099 (0.112)	− 0.068 (0.276)	0.047 (0.456)
Not exposed	− 0.030 (0.634)	0.100 (0.116)	0.068 (0.286)	0.043 (0.496)	0.001 (0.987)	− 0.061 (0.337)	0.122 (0.054)
Body Mass Index (kg/m^2)							
Exposed	**0.214 (< 0.001)**	**0.209 (< 0.001)**	0.111 (0.076)	− 0.004 (0.949)	0.056 (0.370)	**0.197 (0.001)**	**0.187 (0.003)**
Not exposed	− 0.02 (0.964)	**0.179 (0.005)**	0.062 (0.332)	− 0.019 (0.763)	− 0.094 (0.138)	− 0.027 (0.674)	**0.143 (0.023)**
Residence in the municipalities (years)							
Exposed	0.011 (0.862)	0.057 (0.365)	0.036 (0.565)	− 0.037 (0.553)	− 0.019 (0.757)	0.097 (0.119)	0.120 (0.055)
Not exposed	0.056 (0.375)	**0.198 (0.002)**	**0.205 (0.001)**	**0.166 (0.009)**	0.121 (0.056)	0.009 (0.891)	**0.224 (< 0.001)**
Tap water consumption (L/day)							
Exposed	0.090 (0.886)	**− 0.144 (0.021)**	0.112 (0.073)	0.064 (0.309)	0.087 (0.164)	− 0.019 (0.763)	0.043 (0.494)
Not exposed	**− 0.151 (0.016)**	**− 0.183 (0.004)**	− 0.094 (0.136)	− 0.057 (0.369)	0.022 (0.734)	0.045 (0.480)	− 0.119 (0.061)
Well water consumption (L/day)							
Exposed	− 0.011 (0.855)	0.015 (0.813)	0.098 (0.118)	0.058 (0.355)	0.055 (0.378)	0.062 (0.323)	0.097 (0.120)
Not exposed	− 0.016 (0.805)	**0.123 (0.050)**	**0.184 (0.004)**	0.095 (0.134)	0.001 (0.987)	− 0.003 (0.961)	**0.130 (0.039)**

Mann-Whitney and Kruskal-Wallis tests	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFHxS	PFOS
	p[b]	p	p	p	p	p	p
Gender (female/male)							
Exposed	**0.027**	**< 0.001**	**< 0.001**	0.246	0.977	**< 0.001**	**< 0.001**
Not exposed	0.560	**< 0.001**	**< 0.001**	0.140	0.127	**< 0.001**	**< 0.001**
Residence area (urban/suburban/rural)							
Exposed	0.056	**< 0.001**	**0.015**	0.222	0.572	**< 0.001**	**0.002**
Not exposed	0.458	0.767	0.735	0.873	0.546	0.765	0.945
Grow own fruits/vegetables (yes/no)							
Exposed	0.902	0.357	0.215	0.093	0.483	0.151	0.075
Not exposed	0.919	0.250	0.083	0.087	0.628	0.208	**0.035**
Raise own livestock (yes/no)							
Exposed	**0.017**	**0.017**	**0.037**	**< 0.001**	0.387	**0.029**	**< 0.001**
Not exposed	0.671	0.406	**< 0.001**	**< 0.001**	0.785	0.587	**0.043**

[a] Spearman's R correlation coefficient (p value).
[b] p value for Mann-Whitney and Kruskal-Wallis tests for differences between groups.

females for most PFASs both in the E and the NE group (Table 6). In the E group PFOA median concentrations in males (26.07 ng/g) were > 3 times higher than in females (7.88 ng/g), whereas the difference was less pronounced in the NE group (2.04 ng/g in males and 1.27 ng/g in females). This sex-related difference was observed also in other studies (Bartolomé et al., 2017; Calafat et al., 2007a, 2007b; Siebenaler et al., 2017; Steenland et al., 2009). It was hypothesized that the difference may be due to a greater efficiency in the renal elimination of PFASs, in analogy to what was observed in rats (Worley and Fisher, 2015). However, some human biomonitoring studies did not detect sex differences in elimination half-times of perfluoroalkyls (Bartell et al., 2010; Seals et al., 2011).

Differently from sex, age did not seem to strongly affect the levels of PFASs. Although we observed an increasing trend of serum concentrations with age, such correlation was never significant in NE subjects, and resulted to be significant only for PFHpA in E subjects (Table 6). Dependence on age is a quite controversial issue, since some authors reported PFOS and PFOA concentrations significantly higher in older adults than in younger ones (Ji et al., 2012; Lindh et al., 2012); other authors found such correlation limited to females but not males (Fromme et al., 2007), or limited to PFOS (Hsu et al., 2013), whereas in other studies no correlation was found (Yeung et al., 2006; Zeng et al., 2015). It was also reported that the concentrations of some PFASs started to decrease with increasing age (Frisbee et al., 2009; Olsen et al., 2003), possibly reflecting a lower renal re-absorption mechanism in aged individuals.

A direct significant correlation between PFASs and BMI was found in both groups of subjects, more pronounced in the E group than in the NE group (Table 6); this correlation was highly influenced by sex (males had higher BMI than females) and greatly decreased after stratifying by sex.

3.3. Correlation between PFAS serum levels and lifestyle

Serum concentrations of some PFASs increased significantly with increasing years of residence in the municipalities of the NE group (Table 6), in particular for subjects from ULSS 15. The same correlation was observed also in the E group, but never reached the statistical significance.

Concentrations of PFOA, PFNA, PFHxS, and PFOS in the E group differed significantly (Table 6) as a function of residence area type, and resulted to be higher in rural environments. Such difference was not observed in the NE group. However, it should be taken into account that 86% of subjects living in rural areas were from ULSS 5 and, after stratifying by ULSS, the area-related differences in PFAS concentrations lost their statistical significance.

Growing and consuming own vegetables was directly but not significantly associated with PFAS serum concentrations in both E and NE subjects, except for PFOS in NE subjects ($p = 0.035$, Table 6). Raising own livestock was significantly associated with PFHpA, PFOA, PFNA, PFDA, PFHxS, and PFOS concentrations in E subjects (Table 6), and with PFNA, PFDA and PFOS concentrations in NE subjects.

The contribution of food consumption to exposure could not be fully characterized because, in sites with PFAS water contamination, the main source of exposure to these substances is drinking water consumption, as already highlighted in the literature (Brede et al., 2010; Gyllenhammar et al., 2015; Steenland et al., 2009). In addition, the real amount of food consumption is difficult to be quantified on the basis of the self-reported data in a questionnaire that could be subject to over- and under-reporting. Furthermore, for all the 24 food categories included in the analysis, the fraction of locally produced food consumed was very low compared to the fraction purchased from the large distribution. Our analysis highlighted that in the E group there was a significant and direct correlation with six specific food groups out of the 24 considered: cereals and derivatives (PFHpA, PFOA, PFHxS, and PFOS), eggs (PFUdA), shellfish (PFNA), wild fish and game (PFHpA, PFDA, PFUdA, and PFOS), vegetable oils (PFHpA and PFOA), and fruit (PFOA). In the NE population significant direct correlations were observed between the serum levels of PFAS and consumption of: nuts (PFOA, PFNA, PFDA, PFHxS, and PFOS), cheese (PFOS), liver (PFHPA), pork (PFHxS and PFOS), fish (PFNA, PFDA, and PFUdA), shellfish (PFDA and PFUdA) and wild fish and game (PFHpA, PFNA, PFDA, and PFUdA).

Water use in cooking procedures is known to contribute to the amount of PFASs assumed through food (US EPA, 2016b): this particularly holds for those cereals (in particular pasta and rice) which absorb a considerable amount of water during cooking. In fact, the most robust and significant correlation was observed between concentrations of some PFASs (primarily PFOA) and consumption of cereals. Such a correlation resulted not to be influenced by sex.

Wine, beer, and alcohol consumption positively and significantly correlated with PFAS serum levels in E and NE subjects. After stratification by sex, the correlation with beer and alcohol lost significance, whereas the correlation between serum concentrations and wine consumption was still significant, although decreased (increased p values) and involved a lower number of analytes.

3.4. Correlation between PFAS serum levels and drinking water consumption

Tap drinking water consumption was inversely correlated with serum concentrations of some PFASs both in E and NE subjects (Table 6). Considering that tap water was found to be highly contaminated before 2013, this unexpected result for E subjects was strongly influenced by the differences in water consumption in the two ULSS territories of residence of E subjects: in ULSS 5 (the most impacted one) only 41% of subjects declared to drink tap water versus 72% in ULSS 6. After stratification by ULSS, the correlation between tap water consumption and serum PFAS concentrations became direct and significant (for PFOA and PFNA in ULSS 5 and for PFHpA and PFOS in ULSS 6).

The use of well water was very limited in the E group (only six subjects declared to drink well water) while in the NE group 35% of subjects declared to drink well water (residents in ULSS 8 and 15 had the highest well water consumption). In the NE group PFOA, PFNA, and PFOS positively and significantly correlated with well water consumption (Table 6). These findings, together with other results referred to the NE group (highest PFAS serum concentrations related with years of residence in the areas and correlation with growing own vegetables and raising own livestock), may provide an indication of a possible wider diffusion of the water contamination to geographical areas adjacent to those directly affected by drinking water contamination.

3.5. Genotype analysis

Genotype frequencies were calculated (Table 7) and the χ^2 test was applied to investigate whether observed and expected genotype frequencies of SLCO1A2 locus were in Hardy-Weinberg equilibrium. In the population recruited for the project, the Hardy-Weinberg equilibrium was respected (p = 0.219). The existence of the Hardy-Weinberg equilibrium allows to check whether the enrolled population is representative of the actual one, being selected without bias. Indeed, it describes genotype and allele frequencies in a non-evolving population, based on the assumptions that the population is large enough to exclude any genetic drift, gene flow, or natural selection of the studied population.

Table 7
Frequency of SLCO1A2*3 different genotypes in the population's groups.

Genetic polymorphism	Exposed	Not exposed		
	N (%)	*N* (%)	χ^2	p
SLCO1A2*3 (A516C)				
w/w	217 (89)	241 (93)		
w/m	28 (11)	15 (6)		
m/m	0 (0)	3 (1)		
Mutant allele (m) frequency	28 (6)	18 (4)	3.045	0.0810

The allelic frequencies (w = 0.95 and m = 0.05) were comparable with those reported in the literature (Bosó et al., 2014; Laitinen and Niemi, 2011).

The χ^2 test was conducted to investigate the significance of differences in the distribution of genotypes carrying at least one mutant allele (w/m plus m/m individuals) with respect to those carrying two copies of the wild type gene (w/w individuals), assuming a significance cutoff of $p < 0.05$. When comparing E with NE individual alleles distributions there were no statistical differences ($\chi^2 = 3.045$ p = 0810; Table 7).

Kruskal-Wallis and Mann-Whitney tests were performed on subjects enrolled in the study to compare PFAS levels in groups with different genetic makeup (w/w, w/m, and m/m): no significant differences were observed, even when individuals were stratified for exposure (E and NE). The only exceptions were in the NE group in the case of PFPeA, for which the Kruskal-Wallis test showed a significant difference (p value = 0.042, serum concentrations were higher in w/w subjects than in w/m subjects). When considering w/w and w/m groups only, we observed a difference in PFPeA and PFHxA serum levels in the NE group (serum concentrations were higher in w/w subjects than in w/m subjects), for which a p value of 0.011 and 0.047, respectively, was calculated with the Mann-Whitney test. However, the biological meaning of these findings was not clear.

The OATP1A2, whose SNP was here studied, is expressed in the human kidney at the apical membrane of the distal nephron (Lee et al., 2005; Roth et al., 2012). Differently from the role indicated for the corresponding rat transporter OATP1A1 (SLCO1A1), OATP1A2, was shown in vitro to be not directly involved in the cellular uptake of perfluorocarboxylates (Yang et al., 2010; Han et al., 2012). Considering that the studied SNP has been shown to have a different activity when compared to the wild type, at least with some substrates (Table 1), the presence of mutated alleles could have effects (e.g. a change in the affinity for various PFASs, thus recognized as substrates). This could result in OATP1A2 producing a different body burden in the carrier individuals at comparable level of external exposure.

Data show that there are no relationships between the OATP1A2*3 allelic variant and the blood levels of PFOA and other PFASs, at least in the E group. The results suggest that this polymorphism is not able to alter PFAS elimination in a way capable to change the blood levels in the carrier individual. This could represent the in vivo confirmation of the minor role for this transporter in humans compared to rat, as indicated by in vitro results, with a more relevant role for other transporters, such as OAT4 and URAT1 (Urate Transporter 1) (Yang et al., 2010). However, the interpretation of results can be limited by the low number of m/m individuals and the relatively high external exposure: indeed, the only differences were evidenced in the NE group.

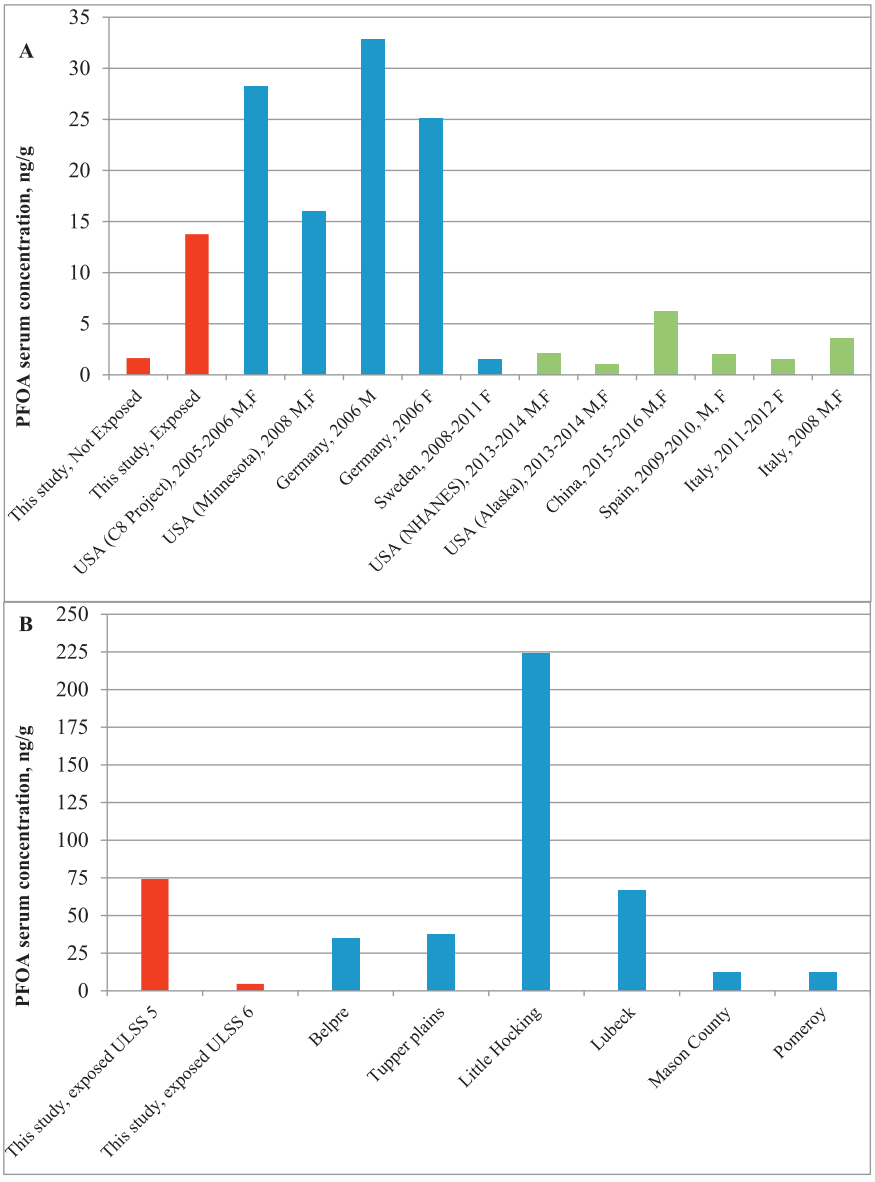

Fig. 2. A. PFOA concentrations (medians, ng/g) found in this study (in red), in other studies of populations exposed to contaminated water (in blue, data from Brede et al., 2010; Frisbee et al., 2009; Gyllenhammar et al., 2015; Kari et al., 2009), and general population groups (in green, data from Bao et al., 2017; Bartolome et al., 2017; Byrne et al., 2017; CDC, 2017; De Felip et al., 2015; Ingelido et al., 2010). Study identifications also show sampling years. B. PFOA concentrations (medians, ng/g) found in ULSS 5 and ULSS 6 from this study (in red), and in the different water districts from the C8 Project (in blue, data from Steenland et al., 2009). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Comparison with results of other biomonitoring studies on populations not occupationally exposed

Fig. 2 (A) shows the serum concentrations of PFOA assessed in this study, in other biomonitoring studies on populations exposed to PFAS contaminated water (Brede et al., 2010; Frisbee et al., 2009; Gyllenhammar et al., 2015; Kari et al., 2009), and in some national and international studies involving the general population (Bao et al., 2017; Bartolomé et al., 2017; Byrne et al., 2017; CDC, 2017; De Felip et al., 2015; Ingelido et al., 2010). On the whole, the data we obtained for NE subjects are in line with the concentrations shown in the figure, and also with concentrations assessed in other studies carried out on the general European population (Glynn et al., 2012; Haug et al., 2009; Nøst et al., 2014; Schröter-Kermani et al., 2013). PFOA serum concentrations of E subjects compare well with data from Minnesota (Kari et al., 2009), are about ten times higher than data from Sweden (Gyllenhammar et al., 2015) and two times lower than data from West Virginia and Ohio (Frisbee et al., 2009) and Germany (Brede et al., 2010). Nevertheless, when comparing our results with those from other studies it has to be taken into account that the E group is very inhomogeneous as the median PFOA serum concentration in ULSS 5 subjects is about 16 times higher than in ULSS 6 subjects (Fig. 2 (B)).

Fig. 2 (B) compares the levels observed in this study with concentrations assessed in subjects participating in the C8 Project (Steenland et al., 2009). This Project involved residents from Ohio and West Virginia communities, who had consumed drinking water contaminated by a nearby PFAS-producing chemical plant. Between 2005 and 2006, 69,030 subjects who lived in six contaminated water districts were enrolled, and PFOA serum levels determined. The differences observed between water districts clearly reflected a different exposure to PFOA, with PFOA serum concentrations decreasing as the distance from the plants increased. Similarly, in our study the significant difference between ULSS 5 and ULSS 6 municipalities clearly reflects a different exposure to PFOA. PFOA serum concentrations in ULSS 6 subjects appear to be lower than concentrations in subjects from all the C8 Project water districts, whereas concentrations observed in ULSS 5 subjects appear to be higher than those from most of the C8 Project water districts (with the only exception of Little Hocking) and also higher than concentrations detected in exposed subjects from Minnesota, Germany, and Sweden.

As to the other PFASs, the majority of biomonitoring studies have analyzed only some of them and mostly refer to general populations

(Calafat et al., 2007a, 2007b; Nøst et al., 2014; Salihovic et al., 2015), rather than to groups exposed to PFAS contaminated water (Brede et al., 2010; Frisbee et al., 2009; Gyllenhammar et al., 2015; Kari et al., 2009). PFAS concentrations measured in this study generally resulted to be comparable to, or lower than, those observed in the aforesaid studies on exposed populations, with the only exception of PFBA and PFBS, for which we observed comparatively higher levels; however, these two analytes were above their respective LOQs only in 60 (12%) and 125 subjects (25%), respectively.

3.7. Toxicological considerations

Due to significant kinetic differences and the presence of mechanisms of action that are not relevant to humans (e.g. effects involving the peroxisome proliferator-activated receptor-α PPAR-α), studies on animals are poorly representative for human extrapolation. This makes the detection of PFAS internal dose particularly relevant, since comparison among studies and in different species should be carried out on such basis, especially when the tissue-specific internal dose is considered, rather than on dietary exposure. A number of human studies were published in the last few years in which both the plasma levels and health effects were measured (US EPA, 2016a, 2016b; Danish EPA, 2015; Health Council of the Netherlands, 2013); these studies especially involved workers or highly exposed subjects as in the C8 Project. Although in many cases the reported PFAS effects were not fully consistent, a re-evaluation of the present tolerable daily intake (TDI) values is currently under way (e.g. in EFSA (European Food Safety Authority)).

The German Human Biomonitoring Commission (Schulz et al., 2007) derived health-related guidance values (HBM I values) on the basis of available toxicological and epidemiological studies from the literature. They represent the concentration of a chemical in a human biological medium, below which there is no risk of adverse health effects, and therefore no exposure reduction measures are necessary (Schulz et al., 2007). HBM I values for PFOA and PFOS in blood plasma were respectively 2 and 5 ng/mL (Apel et al., 2017; Bundesgesundheitsbl, 2016).

In our study, 96% E subjects and 36% NE subjects had PFOA serum concentrations higher than the pertinent HBM I value (notably, 60 subjects exceeded the HBM I values > 10-fold); 79% E subjects and 59% NE subjects had PFOS serum concentrations above the HBM I value (three subjects, one E and two NE, had PFOS levels > 10 times higher than the HBM I value).

These findings indicate a need to reduce exposure and indeed, the Veneto Region implemented a series of risk reduction measures to limit exposure (WHO, 2017), among which the introduction of specific treatments to remove PFASs from the water used for human and animal consumption as well as for irrigation. Since serum concentrations of PFASs reflect the exposure from recent months to several years of accumulation depending on the half-lives of the different compounds, the decrease in body burden will be a slow process. It was reported that the average decrease in PFOA serum levels for individuals enrolled in the C8 Project (residents of Lubeck, West Virginia, and Little Hocking, Ohio, the two districts with the highest impact) was 26% a year after treatment to remove PFOA from the potable water supply began (Bartell et al., 2010). Therefore, it would be necessary to verify over time the effectiveness of the measures implemented to minimize exposure.

4. Conclusions

This study confirmed that water contamination by PFASs in some areas of the Veneto Region resulted in significant years-long exposure of the residing population. Within the group of the exposed subjects, those living in ULSS 5 territory showed the highest PFAS serum concentrations, this finding reflecting the hypothesized different exposure linked to the structure of the public water supply system.

The main factors influencing PFAS serum levels were residence area and the related extent of drinking water contamination. Sex, water consumption, years of residence, and raising own livestock also played a role, while effects of other demographic and environmental factors were relatively weak. No relationship with the genetic trait for the renal transporter studied here was evidenced.

The data obtained in this study provide a baseline characterization of these population groups for subsequent, prospective studies, scheduled to be carried out in the near future.

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