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ORIGINAL ARTICLE

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Perfluoroalkyl substances and bone health in young men: a pilot study

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Abstract

Purpose Perfluoroalkyl substances (PFAS) are a class of endocrine-disrupting chemicals. Toxicological studies indicate that PFAS accumulate in bone tissue and could cause alterations in bone metabolism. The primary objective of this study was to examine the association between PFAS exposure and bone status in a cohort of young men resident in a well-defined area with high PFAS environmental pollution.

Methods Bone status was assessed in 117 subjects aged 18–21 by quantitative ultrasound (QUS) at the heel. Subjects underwent an accurate medical visit. Socio-demographic characteristics, lifestyle, and medical histories were collected. We also verified the interaction between PFAS and hydroxyapatite by computational modelling. The organic anion-transporting peptide (OATP), the putative transporter of PFAS, was evaluated by qPCR in bone biopsies from femoral heads discarded during arthroplasty in three male subjects.

Results Exposed subjects showed significantly lower stiffness index, which resulted in lower *t*-score and higher prevalence of subjects at medium-high risk of fracture (23.6%) compared with controls (9.7%). Data from computational modelling suggested that PFOA exhibits a high affinity for hydroxyapatite, since the estimated change in free energy is in the order of that exhibited by bisphosphonates. Finally, we observed consistent expression of *OATP1A2* gene in primary human osteoblasts.

Conclusions This is the first study reporting increased osteoporosis risk in young men exposed to PFAS and provide preliminary information on molecular mechanisms that could explain this observation, in agreement with previous studies on animal models and humans. However, these results must be interpreted with caution given the cross-sectional study design and the small number of cases.

Keywords pfas · Perfluorooctanoic acid · Endocrine disruptors · Osteoporosis · pfoa · pfos

Introduction

Perfluoroalkyl substances (PFAS) are a class of organic molecules characterized by fluorinated hydrocarbon chains extensively used in industry and consumer products including oil and water repellents, coatings for cookware, carpets, and textiles. PFAS possess unique physical chemical properties due to their amphiphilic structures and their strong carbon–fluorine bonds. Therefore, long chain PFAS are non-biodegradable and bioaccumulate in the environment [1]. PFAS have been found in humans and in the global environment and their toxicity, environmental fate, and sources of human exposure have been a major subject of research. Perfluorooctanoic acid (PFOA) and perfluooctane sulfonate (PFOS) are the predominant forms in human and environmental samples. In addition to their persistence, PFOA and PFOS have been shown to induce severe health consequences, such as neonatal mortality, neurotoxicity, and immunotoxicity. PFAS are found in circulating blood and cord blood and are transferred to the foetus through the placenta during pregnancy or through breast milk during lactation [2–4], acting as endocrine

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disruptors on the foetus and newborns, ultimately leading to developmental defects. The foetus is particularly vulnerable to endocrine disruptors like PFAS that alter hormonal pathways [5, 6]. Toxicological studies indicate that certain PFAS accumulate in bone tissue in the foetus and could cause altered bone development [7]. Few epidemiological studies have reported an inverse relationship between PFAS exposure and bone health [8–11]. Specifically, two cross-sectional studies using samples from the U.S. National Health and Nutrition Examination Survey (NHANES) have found lower bone mineral density with increased exposure to certain PFAS [8, 9]. Only one study has examined the relationship between in utero exposure to PFAS and bone health in offspring during adolescence but only in girls [11], reporting an association between prenatal concentrations of PFAS and reduced bone mass and size in adolescent girls, whereas another study on young obese boys and girls aged 8–12 years reported consistent negative association between all four PFASs studied with bone parameters analyzed by quantitative ultrasound (QUS) [10]. Furthermore, experimental and human autopsy evidence suggests accumulation of PFAS in the skeleton [12–14]. To date, studies investigating the association between PFAS and bone health in young males are lacking. In addition to the aforementioned mechanisms of PFAS toxicity in bone, also the recently well-defined anti-androgenic potential of PFOA and PFOS [15] could play a role in the alteration of testosterone-dependent bone growth in young men.

The primary objective of this study was to examine the association between pre- and post-natal PFAS exposure and bone status by QUS in a cohort of young men resident since birth in a well-defined area with high PFAS environmental pollution in the Veneto region, Italy. QUS assessment correlates with DXA measures of BMD in adults and children, and is particularly suited as a feasible screening option for assessing bone mineral status in young subjects, since it is less expensive, portable, and does not apply ionizing radiation. Second, we aimed to test the hypothesis of a direct interaction between PFAS and hydroxyapatite by computational modelling. Third, we evaluated in bone biopsies the gene expression of the putative transporter of PFAS within target tissues, the organic anion-transporting peptide (OATP), which is typically involved in renal reuptake of these chemicals [16].

Methods

Study population

This study was performed within the annual screening protocol to evaluate male reproductive health in the high schools of Padova and surroundings (Veneto region, northeast of Italy). The aim of this screening is to early diagnose possible risk factors and diseases of the male reproductive system. Here, we report the findings of 117 subjects aged 18–21 who voluntarily agreed to complete the cross-sectional study between October 2017 and December 2018. Included subjects underwent an accurate medical visit including anthropometrics and testicular volume. During the personal interview, information on socio-demographic characteristics, lifestyle, and medical histories were collected. Intake of medication known to affect calcium metabolism, such as calcium tablets, bisphosphonate, and corticosteroids, vitamin D supplementation, and testosterone replacement therapy was recorded. Participants were defined as physically inactive, if they reported less than 1 h of regular physical activity per week. Risky alcohol consumption was defined as daily alcohol intake at or above 30 g/day. 25-hydroxyvitamin D levels were assessed with direct, competitive chemiluminescent immunoassay (LIAISON 25 OH vitamin D TOTAL Assay, DiaSorin Inc.). Written informed consent was obtained from all subjects, and the study was approved by the Research Ethics Committee of the University Hospital of Padova (N. 2208 P). The investigation was performed according to the principles of the Declaration of Helsinki. Participants did not receive any reimbursement. Based on geographical distribution of PFAS pollution, subjects were then grouped on the basis of their residence since birth. In utero exposure represents the most sensitive window to persistent endocrine disruptors and PFAS act on the foetus and newborns, leading to developmental defects [17], for this reason subjects who moved in the exposure area after birth were not included in the study. Based on the degree of pollution, regional authorities [18] have defined an highly-exposed area, the red area, which is the one with the highest PFAS levels. Among the 117 subjects included in the study, 55 were resident in the red zone and 62 lived outside the exposed area (control group).

Quantitative ultrasound (QUS)

QUS measurements were obtained at the calcaneus, the only validated skeletal site for osteoporosis management [19], using the Achilles InSight device (GE Medical Systems Ultrasound, GE Healthcare, USA), a water-based bone ultrasonometer, as described elsewhere [20]. Briefly, the measurements were performed successively on both feet of seated participants by trained and certified examiners. Each examiner performed two QUS measurements on the right foot on each of five volunteers. The system measures the frequency-dependent attenuation of the sound waves (broadband-ultrasound attenuation, BUA) and the speed of sound waves (SOS) as they pass through the heel. A third variable, the stiffness index (SI) was determined. It is a

combination of BUA and SOS. The SI has been shown to produce better effective precision than BUA or SOS alone [21], presumably because it compensates for both variation in heel width, and variation in heel temperature [22]. SI is expressed as a percentage of the mean value in a young adult, and is calculated as follows: $SI = (0.67 \times BUA) + (0.28 \times SOS) – 420$ [23]. Coefficients of variation for the intraobserver variability (BUA 3.11%, SOS 0.44%, SI 2.95%) and the interobserver variability (BUA 3.88%, SOS 0.53%, SI 3.85%) were determined. The system automatically compares individual SI results to values obtained in a normal young reference population, by providing standard deviations (SDs) from reference values. This reference population was provided with the Achilles InSight and contains sex-specific data for Italy. Indices below the reference mean −2.5 SDs from SI were taken to indicate a high osteoporotic fracture risk, indices above the reference mean −2.5 SDs but below the reference mean −1 SD were taken to indicate a medium osteoporotic fracture risk and indices above the reference mean −1 SD were taken to indicate a low osteoporotic fracture risk. Given the low sample size (only three subjects had a *t*-score > −2.5 SD), medium and high risk were pooled together for statistical analyses.

Molecular docking simulation

Three-dimensional molecular models of PFOA (cid: 9554) was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) as pdb file. The three-dimensional structure of a crystal layer of hydroxyapatite proposed by Heinz et al. [24] was also considered. The simulation of PFOA binding to hydroxyapatite was then performed by using a docking procedure based on the Autodock Vina algorithm [25] implemented in the UCSF Chimera 1.12 (https://www.cgl.ucsf.edu/chimera/) molecular modelling software.

Cell culture

The primary explant technique from femoral heads discarded during arthroplasty in three male subjects was used to obtain human osteoblast [26]. Their use for in vitro scientific research does not require ethics approval from the Institutional Review Board. In brief, bone fragments were seeded into T-25 plastic flasks containing DMEM-F/12 (Euroclone) supplemented with 10% FBS (Euroclone), 50 µg/ml ascorbic acid (Sigma-Aldrich), 10-8 M dexamethasone (Sigma-Aldrich), and 10 mM β-glycerolphosphate (Sigma-Aldrich). This allowed osteoblastic precursor cells to migrate from the fragments, differentiate, and proliferate. After confluence, cells were trypsinized and cultured into T-25 plastic flasks containing DMEM-F/12 (Euroclone) supplemented with 10% FBS (Euroclone), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified incubator at 37 °C with 5% CO_2. Third passage cells were used for the evaluation of the osteoblastic phenotype with alkaline phosphatase (ALP) staining. Cytochemical ALP staining was performed directly on cell culture plates using a commercial kit (Sigma-Aldrich) to confirm their osteoblast phenotype. Cells were observed with a light microscope and the presence of ALP was indicated by red precipitates. Only cells with >80% of ALP positivity were used for gene expression analyses.

RNA isolation, cDNA synthesis, and real time PCR

Total RNA was extracted from human osteoblast cells by the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Dnase treatment was performed using the Ambion® TURBO DNA-free™ Kit (ThermoFisherScientific, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA synthesis from total RNA (100 ng) was performed using SuperScript III (Invitrogen, Carlsbad, CA, USA) and random hexamers.

qPCR were performed in a 20 µl final volume containing 20 ng of cDNA, 1× Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA), and a mix of forward and reverse primers (1 mmol/l each). The following primers were used: *OATP1A2* forward 5′-CAGGCAAC TTGTCCTCAAACA-3′ and reverse 5′- AAGGCAGGA TGGGAGTTTCA-3′. Human *GAPDH* was used as a housekeeping gene: forward 5′-TCGACAGTCAGCCGC ATCTT-3′ and reverse 5′-AGGCGCCCAATACGACCAA A-3′. qPCR was performed on thermocycler StepOne plus (Applied Biosystems, Foster City, CA, USA) using the following parameters: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Relative quantification was performed using Delta Delta Ct (ΔΔCt) method [27]. cDNA pool from human brain library (Human MTC panel II, Clontech, Palo Alto, CA, USA) was used as a control.

Statistical analysis

Continuous data are expressed as median (interquartile range). Nominal data are expressed as percentage. The Shapiro–Wilk W test for normality was used to check the distributions of the variables; as almost none of the parameters was normally distributed, and almost all of log-transformed distributions did not satisfy normality, non-parametric statistics was applied. For bivariate analyses, the Mann–Whitney *U* test (continuous data) or chi-square test (nominal data) was used to compare men and women. In a first step, linear regression models adjusted for age, vitamin D, BMI, physical activity, smoking, risky alcohol

consumption, familiarity for osteoporosis, and previous fractures were used to assess the associations between PFAS exposure and SI. In a second step, multinomial logistic regression models with the same adjustment set as mentioned above were performed to analyze the association between PFAS exposure and the QUS-based fracture risk. All statistics were calculated using SPSS (version 23; SPSS, Inc., Chicago, IL). *P* values < 0.05 were considered statistically significant.

Results

General characteristics of the study population stratified by group are given on Table 1. Control subjects had slightly higher physical activity than exposed ones, although not statistically significant. Testicular volume was significantly reduced in the exposed group (Table 1), whereas none of the considered risk factors of osteoporosis differed between groups. Regarding medications know to interfere with bone metabolism, none of the subjects was taking calcium, bisphosphonates, corticosteroids, vitamin D supplementation, or testosterone replacement therapy. With respect to QUS parameters, exposed subjects showed significantly lower SI, which resulted in lower *t*-score and higher prevalence of subjects at medium-high risk of fracture (Table 1).

Linear regression analysis adjusted for confounders (age, vitamin D, BMI, physical activity, smoking, risky alcohol consumption, familiarity for osteoporosis, and previous fractures) confirmed that PFAS exposure was significantly associated with a reduction in SI ($\beta = -0.39$, $p = 0.022$), together with familiarity for osteoporosis ($\beta = -0.31$, $p = 0.031$), but with no significant interaction between group and familiarity ($p > 0.05$). Multinomial logistic regression models adjusted for the same confounders as mentioned above additionally revealed an inverse association between PFAS exposure and QUS-based fracture risk (OR = 2.89, 95% CIs = 1.01–8.22, $p = 0.047$).

Data from docking simulations (Fig. 1) suggested that PFOA exhibits a quite high affinity for hydroxyapatite, since the estimated change in free energy (−6111.5 kcal/mol) upon binding is in the order of the ΔG exhibited by bisphosphonates [28], a class of drugs widely used for the treatment of several metabolic bone disorders associated with increased bone resorption.

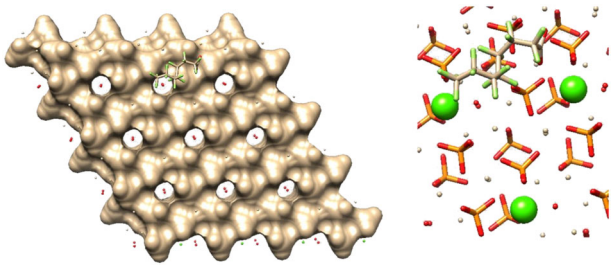

Fig. 1 Docking simulation of PFOA binding to hydroxyapatite tridimensional (left) and molecular (right) structure. The model shows the most likely binding of PFOA within pores between crystals, surrounded by oxygen atoms (green)

Table 1 Characteristics of the study population stratified by PFAS exposure

	All subjects ($n = 117$)	Controls ($n = 62$)	Exposed ($n = 55$)	*p* value[a]
Age (years)	19.0 (18.0–19.0)	19.0 (18.0–19.0)	19.0 (18.0–19.0)	0.662
Physical activity, *n*(%)	76 (65%)	44 (71.0%)	32 (58.2%)	0.105
BMI (kg/m^2)	22.39 (20.7–24.4)	22.1 (20.7–24.6)	22.4 (20.3–24.3)	0.896
Waist circumference (cm)	82.0 (76.0–87.0)	82.5 (76.0–87.1)	81.5 (76.5–85.5)	0.825
25-hydroxyvitamin D (ng/mL)	27.5 (13.9–44.2)	27.1 (13.4–44.0)	28.0 (13.5–45.0)	0.792
Current smoking, *n*(%)	47 (40.2%)	25 (40.3%)	22 (40.0%)	0.561
Alcohol consumption, *n*(%)	11 (9.4%)	6 (9.7%)	5 (9.1%)	0.584
Testicular volume (mL)	14.0 (13.0–16.0)	15.0 (13.0–16.0)	13.5 (12.0–15.0)	**0.010**
Familiarity for osteoporosis, *n*(%)	21 (17.9%)	10 (16.1%)	11 (20.0%)	0.380
Previous fractures, *n*(%)	10 (8.5%)	4 (6.5%)	6 (10.9%)	0.298
Stiffness index	112.5 (95.0–131.8)	114.0 (103.5–133.3)	106.0 (89.5–125.5)	**0.020**
T-score	0.9 (−0.3 to 2.4)	1.1 (0.4–2.6)	0.6 (−0.9 to 2.0)	**0.026**
Fracture risk, *n*(%)				**0.036**
>−1 SD	98 (83.8%)	56 (90.3%)	42 (76.4%)	
≤−1 SD	19 (16.2%)	6 (9.7%)	13 (23.6%)	

Significant *p* values are in bold

[a]Chi-square test (nominal data) or Mann–Whitney *U* test (continuous data) was performed

Finally, by RT-qPCR we observed consistent expression of *OATP1A2* gene in differentiated primary osteoblasts from three human femoral explants discarded during arthroplasty, slightly lower than reference gene expression in human brain cDNA library ($\Delta\Delta Ct = 0.71 \pm 0.02$ and 0.86 ± 0.05, respectively, $p < 0.05$).

Discussion

The results of this pilot study are suggestive of a consistent negative association between PFAS exposure and bone health assessed using calcaneal QUS in young men aged 18–21 years, an age range that up to now has been understudied for this exposure.

Our results are in agreement with limited previous research indicating bone as a target tissue for PFAS toxicity in both animal models and in humans. Foetal bone malformations were reported in rodents with prenatal PFOS exposure [29]. In mice, environmentally relevant doses of PFOS were rapidly deposited in bone [12]. In a recent study prenatal and lactational exposure to PFOA in mice resulted in PFOA accumulation in long bones, detectable even 17 months after the exposure, and in altered bone geometry and decreased bone mineral density [7]. Taken together, these studies suggest that PFAS are deposited in bone and may induce osteotoxicity. In human autopsy studies, PFAS were sequestered in bone, with PFOA being predominant [13], and a role for organic anion transporter peptides has been suggested, as for other districts with known PFAS accumulation [5]. We report OATP1A2 gene expression in human bone samples for the first time, suggesting its involvement in aforementioned PFAS accumulation in bone tissues. However this aspect clearly requires further experimental evidence.

Using cross-sectional data from NHANES, two studies reported inverse associations between PFAS exposure and bone health in women [8, 9]. These observations were also extended to younger ages, as reported in in adolescent girls, where prenatal concentrations of some PFAS were associated with reduced bone mass and size [11], suggesting an early manifestation of PFAS osteotoxicity. Indeed also in young obese children, a negative association between PFAS and bone density was reported [10]. However, to our knowledge this is the first study to investigate the role of PFAS exposure on bone health in an adult male population.

Although different mechanisms have been proposed to explain the aforementioned reduction of bone density in exposed subjects, mainly mediated by an indirect interference of PFAS on hormonal regulation of bone health, we propose a direct mechanism of interference on the main component of bone matrix that is hydroxyapatite. Although preliminary, our computational results suggest a direct binding of PFOA within hydroxyapatite crystals, with a binding affinity within the same range of bisphosphonates, a class of drugs widely used in the treatment of several metabolic bone disorders associated with increased bone resorption. This observation clearly requires further investigation in cellular and animal models.

There are several study limitations, prominent being a small sample size. Our study population included only Caucasian subjects and the results may thus not apply to other ethnicities. Further biochemical analyses on osteoporotic risk factors, such as fasting glucose and direct calcium measure rather than only calcium intake should also be performed. Moreover, subjects showing increased risk for osteoporosis by QUS assessment, should be confirmed by DXA measurements to be clinically relevant. In addition, due to our cross-sectional study design, we cannot conclude causality of any relationships. Finally we categorized exposed subjects only by indirect means based on long-lasting and comparable exposure time depending on the residence within a well-defined area with consistent PFAS exposure [30]. Direct serum quantification of PFAS would be preferable. Nonetheless, the same study population has already been screened for PFAS exposure in another study by our group [15], showing consistent serum PFOA levels in subjects from exposed area.

Besides, methodical differences to assess bone quality may apply. Unfortunately, we do not have DXA measurements. Although DXA is considered the gold standard for BMD assessment [31], it is costly and includes radiation exposure, although at very low negligible levels. In contrast QUS is low-cost, convenient, and radiation-free [32], characteristics which are particularly appropriate to assess bone health in screening campaigns on young subjects, although it is less precise and does not provide information on bone mineral density or content. QUS assessment indeed provides bone structural information, reflecting its elasticity [33]. Conflicting results from US and DXA are neither surprising nor infrequent, and are not necessarily indicative of an error. Rather, QUS parameters are independent predictors of fracture risk, as they are affected by other bone characteristics. Although it is not a diagnostic tool, QUS is considered a feasible initial screening option for assessing bone mineral status in children and young subjects. QUS predicts fracture risk [34] and correlates with DXA measures of BMD in both adults [35] and children [33, 36, 37]. Although there is some evidence that QUS can predict fracture in men, there is not enough evidence to advocate adding QUS to bone density measured by DXA to predict fracture, on the other hand the International Society for Clinical Densitometry concludes that QUS can be used itself to predict fracture in men older than 65 [19].

The majority of previous studies [8, 9, 11] used DXA to assess BMD, whereas the present and another study [10] applied QUS.

In conclusion, this is the first study reporting increased osteoporosis risk in young men exposed to PFAS. These results are in agreement with previous studies in animal models and humans reporting a significant reduction in bone mineral density associated with PFAS exposure. Altogether these results clearly require further investigation and must be interpreted with caution given the cross-sectional study design and the small number of osteoporosis cases in the study population.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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