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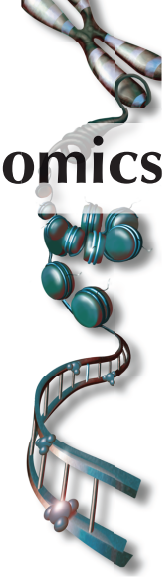
Epigenomics



Per- and polyfluoroalkyl substances, epigenetic age and DNA methylation: a cross-sectional study of firefighters

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Background: Per- and polyfluoroalkyl substances (PFASs) are persistent chemicals that firefighters encounter. Epigenetic modifications, including DNA methylation, could serve as PFASs toxicity biomarkers. **Methods:** With a sample size of 197 firefighters, we quantified the serum concentrations of nine PFASs, blood leukocyte DNA methylation and epigenetic age indicators via the EPIC array. We examined the associations between PFASs with epigenetic age, site- and region-specific DNA methylation, adjusting for confounders. **Results:** Perfluorohexane sulfonate, perfluorooctanoate (PFOA) and the sum of branched isomers of perfluorooctane sulfonate (Sm-PFOS) were associated with accelerated epigenetic age. Branched PFOA, linear PFOS, perfluorononanoate, perfluorodecanoate and perfluoroundecanoate were associated with differentially methylated loci and regions. **Conclusion:** PFASs concentrations are associated with accelerated epigenetic age and locus-specific DNA methylation. The implications for PFASs toxicity merit further investigation.

Lay abstract: Per- and poly-fluoroalkyl substances (PFASs) are a group of toxic chemicals that populations around the world are widely exposed to through contaminated water and consumer products. Firefighters can also be exposed to PFASs from occupational practices. Epigenetic modifications, including DNA methylation, regulate gene expression. It can be modified by environmental exposures such as PFASs, which contribute to the development of diseases including cancer. We measured the concentrations of nine PFASs in samples from firefighters and profiled DNA methylation across the genome. Three PFASs were linked with accelerated epigenetic age, a marker associated with many diseases. Four PFASs were associated with altered DNA methylation levels at specific genes. These results may indicate how PFASs are harmful to health and merit further exploration.

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Per- and polyfluoroalkyl substances (PFASs) are ubiquitous in households, workplaces and the environment around the world [1,2]. Owing to environmental persistence and growing evidence of toxicity, manufacturing of legacy long-alkyl chain PFASs (e.g., perfluorooctanoate [PFOA] and perfluorooctane sulfonate [PFOS]) has been eliminated or greatly reduced in many countries, including the USA, in the past two decades. Even so, PFASs exposure remains a persistent issue as replacement PFASs are increasingly in use and legacy long-alkyl chain PFASs remain in the environment, in older household products and are still newly manufactured in some parts of the world [3,4]. Common sources of exposure, for the general US population, include contaminated drinking water and the use of PFASs-containing products such as cookware, carpet and food packaging [5–8].

Firefighters may experience higher exposures to PFASs than the general population. First, exposure to heat can mobilize PFASs and its precursors that are found in household products such as carpet [9–12]. Second, PFASs are ingredients of Class B aqueous film forming foams (AFFFs) used for the suppression of liquid fuel fires [9,13,14]. Firefighters in departments using Class B foams are thus exposed when using AFFFs and potentially through consumption of contaminated ground water at training sites [15–19]. Third, PFASs are used for oil and water resistant coatings on firefighter turnout gear thus increasing potential for associated dermal, inhalation or ingestion exposure to station dust from these textiles, although the extent of actual absorption into firefighters remains unclear [20]. Adverse health effects reported in firefighters [21–23], including risks for certain cancers [24–27], are similar to health effects associated with PFASs exposure in other occupational groups and the general population. Adverse health outcomes associated with PFASs exposure in other populations include elevated cholesterol [28–30], altered immune response [31], respiratory disease [32,33] and urogenital cancers [34,35].

The epigenome can be responsive to environmental exposures, both globally and at a specific gene level. DNA methylation is a key epigenetic regulator that involves the addition of a methyl group to the 5-carbon of cytosine residues specifically at CpG dinucleotides and typically results in gene repression [36]. DNA methylation patterns are informative for both estimations of epigenetic age and for examining regulation of specific genes with relevant biological functions. Adult DNA methylation profiles are associated with myriad environmental exposures including smoking, psychosocial stress, metals and air pollution [37–40]. Epigenetic alteration could serve as a subtle indicator, or effect biomarker, of toxicity that is present long before the development or diagnosis of overt disease [41]. The epigenome changes with age as physiological function changes and declines over the lifespan. The study of DNA methylation across the lifespan has led to the development of several epigenetic age biomarkers, also referred to as epigenetic clocks [42]. Epigenetic clocks can be used to infer whether epigenetic age is accelerated compared to chronological age. Importantly, recent evidence has suggested that these clocks are more accurate at estimating an individual's 'biological age' [43]. This acceleration has been linked to cancers, mortality, cardiovascular disease and additional health-related endpoints in multiple cohort studies [44–46]. Research on occupational and environmental factors that accelerate epigenetic aging is limited (e.g., shiftwork), and to our knowledge, associations between PFASs and epigenetic aging have not been evaluated [42,47].

In our pilot research, we observed associations between years of firefighting and altered DNA methylation as well as altered miRNA expression [48–50], although these studies did not include the assessment of PFASs exposures. The *in vitro* expression of 20 genes involved in lipid metabolism, xenobiotic metabolism and antioxidant stress response was altered in human hepatocytes following treatment with multiple individual PFASs including six quantified in the current study [51]. DNA methylation alteration by PFASs may be a mechanism leading to these expression differences. A recent review on PFASs and epigenetic changes provides reasonable evidence for a link between PFASs and altered DNA methylation in human, animal and *in vitro* studies [52]. However, most epidemiological studies on this topic have been focused on prenatal exposures with the following exceptions. Among 685 adults highly exposed to PFOA through contaminated drinking water, serum PFOS but not PFOA, perfluorohexane sulfonate (PFHxS) or perfluorononanoate (PFNA) was associated with increased methylation of LINE-1 repetitive elements, considered to be a marker of widespread epigenetic changes [53]. Two epigenome-wide association studies reported dozens of loci associated with exposure among Swedish women highly exposed to PFOA, PFOS and PFHxS through drinking water [54] and among Dutch men exposed to PFOS [55]. However, these studies were limited by relatively small sample sizes (n = 59 and 34, respectively) and a focus on populations that were highly exposed to a few PFAS.

Table 1. Per- and poly-fluoroalkyl substances concentrations in serum samples from US firefighters recruited between 2016 and 2019 (ng/ml, n = 197).

Abbreviation	Full name	% above LOD	Geometric mean (95% CI)	25th percentile, 75thpercentile	NHANES‡, geometric mean (95% CI)
PFHxS	Perfluorohexane sulfonate	100	2.50 (2.29–2.74)	(1.70, 3.80)	1.22 (1.11–1.34)
n-PFOA	Linear perfluorooctanoate	100	1.79 (1.68–1.89)	(1.40, 2.20)	1.50 (1.41–1.61)
Sb-PFOA	Sum of branched isomers of perfluorooctanoate	31.0	†	(<LOD, 0.10)	†
n-PFOS	Linear perfluorooctane sulfonate	100	4.02 (3.74–4.32)	(3.00, 5.80)	3.38 (3.07–3.73)
Sm-PFOS	Sum of perfluoromethylheptane sulfonate isomers	100	2.06 (1.91–2.23)	(1.40, 3.10)	1.52 (1.42–1.62)
PFNA	Perfluorononanoate	98.5	0.44 (0.41–0.48)	(0.30, 0.60)	0.59 (0.55–0.64)
PFDA	Perfluorodecanoate	99.0	0.23 (0.22–0.25)	(0.20, 0.30)	0.16 (0.14–0.18)
PFUnDA	Perfluoroundecanoate	66.0	0.12 (0.11–0.13)	(<LOD, 0.20)	†
MeFOSAA	2-(N-methyl-perfluorooctane sulfonamido) acetate	27.9	†	(<LOD, 0.10)	†

†Not calculated: proportion of results <LOD was too high to provide a valid result.
‡Results are from 1640 adults (aged 20+ years) sampled in the 2015–2016 cycle.
LOD: Limit of detection; NHANES: US National Health and Nutrition Examination Survey.

In the current study, we have assessed whether PFASs serum concentrations are associated with epigenetic age biomarkers and locus-specific blood leukocyte DNA methylation in a pilot sample of 197 firefighters from three cities in the USA. We hypothesized that serum PFASs concentrations will be associated with accelerated epigenetic aging and differences in DNA methylation at genes with biological functions relevant to PFASs toxicity, assessed via the Infinium MethylationEPIC (Illumina, CA, USA).

Methods

Study population

This pilot study included participants from two larger studies focused on assessing cancer risk factors among firefighters: the first is a 3-year research project with the University of Arizona working in partnership with the Tucson Fire Department (TFD), and the second is the prospective, multicenter Fire Fighter Cancer Cohort Study [56] involving multiple fire departments and universities as well as the National Institute for Occupational Safety and Health (NIOSH) at the US Centers for Disease Control and Prevention (CDC). Incumbent firefighters were recruited for this study from career fire departments in Arizona (from 2016 to 2018, n = 138), California (2019, n = 52) and Massachusetts (2018, n = 7). Inclusion criteria for enrollment in the current study included being an active-duty firefighter (including emergency medical responder) who responds to fires as part of normal duties. All study procedures were approved by the institutional review boards (IRBs of the University of Arizona (IRB approval no. 1509137073) and the University of Miami (IRB approval no. 20170997). The informed consent process included the research team delivering an in-depth explanation of the study design and potential risks and responsibilities. The analysis of de-identified specimens at the CDC laboratory was determined not to constitute engagement in human subjects research.

Data & sample collection

Participants answered a questionnaire to collect information on the following: demographics; occupation; health factors, such as previous cancer diagnosis, body weight and height; duration of service as a firefighter including at current and previous departments and current and previous behaviors, such as tobacco use. Blood samples were collected during the day by qualified phlebotomists in one 10-ml red top tube (for PFASs quantification) and one 6-ml dipotassium ethylene diamine tetraacetic acid tube (for isolation of DNA; BD, NJ, USA). The red top tube was centrifuged at 1000–1300×*g* for 15 mins, and the serum was dispensed into 1-ml aliquots in polypropylene cryovials. Blood was stored frozen (temporarily at -20°C followed by long-term storage at -80°C) until use [49].

PFASs quantification

Frozen serum samples were shipped to the CDC National Center for Environmental Health (NCEH) laboratory overnight on dry ice according to International Air Transportation Authority guidelines. Concentrations of nine PFASs (Table 1) in 0.05 ml of serum were quantified by on-line solid phase extraction liquid chromatography-

isotope dilution tandem mass spectrometry according to the methodology described before [57]: 2-N-methyl-perfluorooctane sulfonamide acetate (MeFOSAA), PFHxS, PFNA, perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnDA), linear perfluorooctanoate (n-PFOA), sum of branched PFOA isomers (Sb-PFOA), linear perfluorooctane sulfonate (n-PFOS) and sum of perfluoromethylheptane sulfonate isomers (Sm-PFOS). The CDC laboratory is certified by the Health Care Financing Administration to comply with the requirements set forth in the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88) and is recertified every 2 years. CDC staff performed the analytical measurements following strict quality control/quality assurance CLIA guidelines, including participation in external quality assessment schemes to demonstrate accuracy and precision. Furthermore, along with the study samples, each analytical run included high- and low-concentration quality control materials, analytical standards, and reagent blanks to assure the reliability of the data [57]. The limit of detection (LOD) for all PFASs was 0.1 ng/ml. Concentrations below the LOD were replaced with LOD/sqrt(2) for the statistical analyses.

DNA methylation via EPIC

DNA was isolated from blood leukocytes, and quantity was measured via a QuantiFluor dsDNA System (Promega, WI, USA) or a Qubit Fluorometer (Thermo Fisher Scientific, MA, USA). Approximately 500 ng per sample was bisulfite converted using Zymo kits following the manufacturer's recommendations for downstream Infinium array analysis. DNA methylation was quantified at >850,000 CpG sites throughout the genome using the Infinium MethylationEPIC array [58]. Laboratory and data processing methods were previously described [59]. Briefly, samples were randomized across chips, hybridized and scanned in batches at the University of Utah DNA Sequencing and Genomics Core Facility (two batches) or the University of Michigan Advanced Genomics Core (three batches). Raw image files were read with the R package minfi [60], and quality control and normalization occurred using the package ENmix [61] for data from all batches together. Probes were removed if at least 5% of samples were not detected (p-value $>1 \times 10^{-16}$ compared to background). Background correction was performed with noob and dye bias correction with regression on logarithm of internal control (RELIC) followed by quantile normalization [62]. Probes that are known to be cross-reactive [62], have SNPs in the CpG or single-base extension site (based on probe annotation information from the manufacturer), or are on X and Y chromosomes were excluded with a final 740,842 CpG sites included in downstream analyses. Samples passing all quality control measures that also had PFASs quantified in serum and covariate data (n = 197) were included. Surrogate variable analysis (SVA) was performed using the intensity values from the non-negative control probes to create variables representing technical variation influencing the DNA methylation data [63]. Three principal components (PCs) from this analysis explained 92% of the technical variance in the data and were used as covariates in downstream models.

Cell type estimates

Because blood is a heterogeneous tissue, the proportion of cell types was measured using the methods based on Infinium methylation data. Specifically, we estimated proportions of $CD4^+$ T cells, natural killer (NK) cells, B cells, monocytes and granulocytes using an established algorithm designed for Infinium data [64]. We also estimated the relative abundance of three additional blood cell types – plasma blasts, $CD8^+CD28^-CD45RA^-$ T cells, and naive $CD8^+$ T cells – using the software developed by Horvath *et al.* [65].

Epigenetic age indicators

Indicators of epigenetic age developed using Infinium methylation data, referred to as epigenetic clocks, were obtained with the software by Horvath *et al.* [65]. We uploaded EPIC data to the New Methylation Age Calculator (https://dnamage.genetics.ucla.edu/new) to calculate epigenetic age biomarkers and selected seven for downstream analyses. We selected the original Horvath clock which was designed to be agnostic to the source tissue type [65], and the skin-blood clock, a more robust estimator for skin, blood, or saliva-derived sample types [66]. The Hannum clock was designed for blood samples [67]. PhenoAge and GrimAge were also selected; these are considered better predictors of all-cause mortality, cancers and other adverse health outcomes compared to the original epigenetic clocks [46,68,69]. For Horvath, Hannum, skin-blood, PhenoAge and GrimAge, we used the residuals after regressing each on chronological age in all statistical analyses. This is the recommended procedure, and positive values indicate accelerated epigenetic/biological aging compared to chronological age. We also calculated intrinsic epigenetic age acceleration (IEAA) and extrinsic epigenetic age acceleration (EEAA). IEAA is the residual after regressing the Horvath clock on chronological age and cell type estimates; it is considered an indicator of cellular aging

independent of cellular composition. By design, EEAA captures cellular methylation changes and extracellular changes in blood cell composition, and the method to obtain EEAA was previously described [45].

Statistical analysis

All analyses were performed in the R Project for Statistical Computing, version ≥ 3.6.4. Descriptive statistics were first calculated for covariates (i.e., demographics), PFASs serum concentrations and epigenetic age biomarkers. Variable distributions were examined, and natural-log transformed to achieve normality if necessary. To determine potential confounders in the relationship between PFASs concentrations and DNA methylation, we first examined relationships between PFASs and covariates (age, gender, race, ethnicity, smoking history, BMI, fire department, years of firefighting and estimated blood cell type proportions) using Pearson's correlations (ln-transformed PFASs and continuous variables), two-sample t-tests (ln-transformed PFASs and two-category variables) or ANOVA (ln-transformed PFASs and three-category variables). The Welch's test was used instead if variances were unequal between groups. We next used Singular Value Decomposition analysis to identify technical and biological covariates that correlate with variation in the DNA methylation data as previously described [59].

We modeled PFASs with at least 30% of samples having detectable concentrations as continuous variables following natural-log transformation to achieve a normal distribution (PFHxS, n-PFOA, Sm-PFOS, n-PFOS, PFNA and PFDA). We modeled the other PFASs (MeFOSAA, PFUnDA and Sb-PFOA) with >30% of samples with nondetectable concentrations as categorical variables (above LOD vs below LOD).

We used a three-tiered analysis approach to investigate associations between PFASs and DNA methylation using: seven epigenetic age indicators; a hypothesis-driven investigation of genes whose expression was associated with PFASs *in vitro* and an epigenome-wide approach using all loci available from the EPIC data. We regressed each of the seven epigenetic age indicators separately over each PFASs. These models adjusted for potential confounders in the relationship including age, gender, race (white vs all other, given that >95% were white), cell type estimates (granulocytes, $CD4^+$ T cells, plasmablasts, $CD8^+$ naive T cells, and $CD8^+CD28^-CD45RA^-$ T cells) and PCs representing technical variation from the EPIC analysis. We also ran models without cell type adjustment. Results from the cell-free model were similar and are not reported here.

In the hypothesis driven approach, we selected 20 genes involved in lipid and cholesterol metabolism, xenobiotic metabolism, and antioxidant stress response that had gene expression changes *in vitro* following exposure to at least three of the following PFASs that were also measured in our study: PFHxS, PFOA, PFOS, PFNA, PFDA and PFUnDA [70]. Genes were selected from these pathways because PFOA and PFOS exposure is associated with perturbation in serum lipids [29,30]. Alteration of xenobiotic metabolism and oxidative stress signaling pathways have been described in human hepatocytes treated with PFASs, as well as in rodent models of PFOA exposure [71,72]. The EPIC array included 433 CpG sites annotated to the 20 genes. In the exploratory epigenome-wide approach, we included 740,842 CpG sites that passed quality control.

For the hypothesis-driven and epigenome-wide approaches, we fit linear regression models of beta values (proportion methylated) for each CpG site with each PFAS. An empirical Bayesian method in the limma R package [73,74] was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic prior to significance calling. The primary statistical model in both approaches included three PCs representing technical variability on the array and potential confounders in the relationships between PFASs and DNA methylation: gender, race, ethnicity, age and cell type estimates (granulocytes, plasmablasts, $CD8^+$ naive T cells, and $CD8^+CD28^-CD45RA^-$ T cells). As sensitivity analyses, we also ran a model with the same covariates plus time working as a firefighter (in years) which may serve as a proxy for cumulative occupational exposures. We tested inclusion of fire department in the model and this increased p-value inflation, thus it was excluded. In the hypothesis-driven approach, we considered associations with p-values <0.0025 to be statistically significant. This accounts for modeling data from 20 different genes; sites within the same gene are correlated with one another to varying degrees. In the exploratory approach, CpG sites associated with PFASs concentrations were considered statistically significant at the epigenome-wide association study p-value of $<9. \times 10^{-8}$ recommended for the EPIC array which is based on the number of comparisons but also takes into account correlation between some of the CpG sites on the array [75]. Given the exploratory nature of this part of the study, we also report loci with a less-stringent cutoff, a false discovery rate adjusted p-value (also called a q-value) [76] <0.2 in the Supplementary Material.

We performed further analyses for the widely detected PFASs with evidence of differential methylation in the exploratory analysis (at least one CpG site associated with PFASs at $q < 0.2$). n-PFOS, PFNA and PFDA met

the criteria. We performed a pathway analysis with the results from the main model to determine whether the top 10,000 CpG sites by raw p-value were enriched in certain biological pathways or gene-function concepts (all p-values were <0.02). We used the gometh function in the missMethyl package, and ran pathway analysis separately for Kyoto Encyclopedia of Genes and Genomes (KEGG; n = 340) and gene ontology (GO; n = 22,768) terms [77]. We report gene concepts enriched at $p < 0.001$ according to missMethyl; redundant concepts were removed using REVIGO [78]. We also used DMRcate to identify differentially methylated regions (DMRs) consisting of at least two consecutive CpG sites, adjusting for the same covariates. This analysis applies Gaussian smoothing to test statistics from per-CpG site analysis using a bandwidth of 1000 bp. Smoothed test statistics are modeled with the Satterthwaite method, and then p-values are computed and adjusted using the Benjamini–Hochberg method (q-values). A relaxed significance cutoff of $q < 0.2$ was set as the threshold for considering individual CpG sites significant. Neighboring significant CpG sites are then agglomerated into DMRs [79].

While not a primary aim of the study, PFASs are reported to be immune disruptors [80], and blood leukocytes are key players of the immune system. We assessed associations between serum concentrations of each PFASs and ten cell type estimates from the samples. We performed unadjusted analyses and multiple linear regression adjusting for potential confounders in the relationship between PFASs and cell types (sex, race, age and PC1 for EPIC batch). We performed two additional models adjusting for the same covariates and smoking status or years working as a firefighter.

Results

Study population characteristics

The study sample consisted of 197 incumbent firefighters from three fire departments who had PFASs serum measurements and DNA methylation data that passed quality control checks. Table 2 displays descriptive statistics for the study sample. Participants had an average of 15 years of firefighting experience (standard deviation [SD] = 9). The sample was primarily male (89.3%), non-Hispanic (84.8%) and white (95.4%).

PFASs exposure assessment

Serum concentrations of nine PFASs were quantified in serum. Of the nine PFASs, we detected PFHxS, n-PFOA, Sm-PFOS and n-PFOS in all participants and PFNA and PFDA in >98% of participants. We detected MeFOSAA, PFUnDA and Sb-PFOA in 28%, 66% and 31% of samples, respectively. When comparing concentrations with those from adults sampled for the representative US National Health and Nutrition Examination Survey (NHANES; 2015–2016 cycle), geometric means among firefighters for PFHxS, n-PFOA, n-PFOS, Sm-PFOS, PFDA and PFUnDA were higher, yet not substantially so, compared to the general population (Table 1). Most PFASs were highly positively correlated with one another. Sm-PFOS, n-PFOS and PFNA were also positively correlated with age. PFHxS, PFNA, n-PFOA, Sm-PFOS, and n-PFOS were all significantly higher in males compared with females (Supplementary Table 1; t-test $p < 0.05$). The three fire departments had different average concentrations of PFHxS, PFNA, Sm-PFOS and n-PFOS (ANOVA $p < 0.001$).

PFASs & epigenetic age

In adjusted linear regression, there were positive associations between PFASs (PFHxS, n-PFOA, Sb-PFOA, n-PFOS and Sm-PFOS) with nearly all seven epigenetic age biomarkers, yet not all of the relationships were statistically significant (Table 3). These associations were statistically significant for PFHxS with three clocks (EEAA, Hannum and skin-blood), n-PFOA with six clocks (all except GrimAge) and Sm-PFOS with two clocks (IEAA and Horvath). There were no statistically significant associations between the other PFASs, with the exception of inverse associations between PFDA and PFUnDA with GrimAge ($p < 0.05$).

PFASs & DNA methylation: hypothesis-driven approach

We selected 20 genes that had shown altered expression following acute PFASs treatment in cultured primary human hepatocytes [70]. When examining the associations between PFASs and blood leukocyte DNA methylation at 433 CpG sites annotated to these genes, five PFASs were associated with sites within seven of these genes ($p < 0.0025$; Table 4). PFHxS was associated with increased methylation in sites within *ABCA1*, *HMGCS1* and *NR1L2*; PFNA was also positively associated with methylation at a CpG site in *ABCA1*. Sb-PFOA was positively associated with CpG sites in *PPARG* and *CD36*, while n-PFOA was inversely associated with methylation at one CpG site in *ACOT2*. PFUnDA was associated with methylation at sites in *PPARG* and *FASN*.

Table 2. Study population characteristics.

Characteristics	n (%)
Gender	
Male	176 (89.3)
Female	21 (10.7)
Ethnicity	
Hispanic	30 (15.2)
Non-Hispanic	167 (84.8)
Race	
Caucasian	188 (95.4)
All other	9 (4.6)
Fire department	
Department A	138 (70.1)
Department B	52 (26.4)
Department C	7 (3.6)
Smoking history	
Never	151 (76.6)
Past	32 (16.2)
Missing/not reported	14 (7.1)
	Mean ± SD
Age (years)	38.6 ± 9.7
BMI (kg/m^2)	26.94 ± 3.35[†]
Years of firefighting	15 ± 9
Estimated cell type proportions (%)	
Granulocytes	57.5 ± 9
CD4$^+$ T cells	13.7 ± 4.8
CD8$^+$ T cells	10.4 ± 4.3
Monocytes	7.7 ± 2.5
B cells	5.3 ± 2.3
Natural killer cells	6.3 ± 2.9
Estimated relative cell abundance	
Plasmablasts	1.86 ± 0.25
CD8$^+$CD28$^-$CD45RA$^-$	5.6 ± 3.52
CD8$^+$ naive cells	208.19 ± 42.33
CD4$^+$ naive cells	679.64 ± 102.59
Epigenetic age indicators (years)[‡]	
Horvath	38.95 ± 9.14
Hannum	32.11 ± 8.65
PhenoAge	24.43 ± 9.95
Skin-blood	46.53 ± 10.32
GrimAge	62.18 ± 7.82
Epigenetic age indicators[‡]	
IEAA	0.51 ± 4.63
EEAA	-0.01 ± 5.03
Horvath – residual	0.79 ± 5.19
Hannum – residual	-0.05 ± 3.76
PhenoAge – residual	0.09 ± 4.66
Skin-blood – residual	-0.07 ± 3.28
GrimAge – residual	-0.24 ± 2.56

[†]Five results missing.
[‡]Mean (SD) are shown for the values calculated by each epigenetic clock as well as for the residuals after regressing each clock on chronological age. The residuals are what is used in statistical models since chronological age explains a large proportion of the variation in these clocks.
EEAA: Extrinsic epigenetic age acceleration; IEAA: Intrinsic epigenetic age acceleration; SD: Standard deviation.

Table 3. Associations between per- and poly-fluoroalkyl substances serum concentrations and seven epigenetic age indicators ($p < 0.05$).

PFAS	Model results	IEAA†	EEAA	Horvath	Hannum	PhenoAge	Skin-blood Clock	GrimAge
PFHxS	Estimate (SE)	0.77 (0.5)	1.05 (0.44)	0.93 (0.5)	0.97 (0.4)	0.58 (0.57)	1.13 (0.4)	0.49 (0.27)
	p-value‡	0.128	0.017	0.066	0.017	0.309	0.005	0.069
n-PFOA	Estimate (SE)	2.12 (0.71)	1.57 (0.63)	2.28 (0.71)	1.45 (0.58)	1.62 (0.82)	1.71 (0.58)	0.16 (0.39)
	p-value	0.003	0.014	0.002	0.014	0.049	0.003	0.686
Sb-PFOA§	Estimate (SE)	0.63 (0.69)	0.09 (0.60)	0.70 (0.69)	0.08 (0.56)	0.23 (0.78)	0.49 (0.55)	-0.3 (0.36)
	p-value	0.358	0.881	0.310	0.881	0.767	0.371	0.414
n-PFOS	Estimate (SE)	0.8 (0.68)	0.83 (0.6)	1.04 (0.68)	0.77 (0.55)	-0.36 (0.77)	0.46 (0.55)	0.65 (0.36)
	p-value	0.239	0.165	0.128	0.165	0.641	0.403	0.071
Sm-PFOS	Estimate (SE)	1.69 (0.65)	0.97 (0.57)	1.85 (0.64)	0.89 (0.53)	-0.18 (0.74)	0.88 (0.53)	0.6 (0.34)
	p-value	0.009	0.092	0.004	0.092	0.810	0.096	0.085
PFNA	Estimate (SE)	0.03 (0.64)	-0.11 (0.56)	0.09 (0.64)	-0.1 (0.52)	-0.73 (0.72)	-0.34 (0.51)	-0.18 (0.34)
	p-value	0.962	0.842	0.893	0.842	0.313	0.510	0.592
PFDA	Estimate (SE)	-0.63 (0.7)	-0.18 (0.61)	-0.43 (0.7)	-0.16 (0.57)	-0.6 (0.79)	-0.71 (0.56)	-0.91 (0.36)
	p-value	0.366	0.773	0.537	0.773	0.445	0.210	0.013
PFUnDA§	Estimate (SE)	0.09 (0.62)	-0.72 (0.54)	0.03 (0.62)	-0.67 (0.5)	-0.47 (0.7)	-0.57 (0.5)	-0.76 (0.33)
	p-value	0.886	0.185	0.965	0.185	0.503	0.260	0.020
MeFOSAA§	Estimate (SE)	0.45 (0.67)	-0.87 (0.59)	0.47 (0.67)	-0.81 (0.54)	-0.69 (0.76)	-0.27 (0.54)	0.32 (0.36)
	p-value	0.505	0.139	0.488	0.139	0.364	0.613	0.363

†The outcomes are as follows: IEAA and EEAA. The rest are residuals after regressing the clock (Horvath, Hannum, PhenoAge, skin-blood or GrimAge) over chronological age. Positive indicates 'faster' aging.
‡Estimates for the association between each PFASs with each epigenetic age indicator are shown. Multivariable linear regression models adjusted for age, gender, race (white vs all other), PC1, PC2, PC3, neutrophils, $CD4^+$ T cells, plasmablasts, $CD8^+$ naive T cells and $CD8^+CD28^-CD45RA^-$ cells.
§PFASs modeled as categorical variables comparing detectable (values above the limit of detection) to nondetectable concentrations because >30% of samples had concentrations below the limit of detection. All other PFASs concentrations were ln-transformed.
EEAA: Extrinsic epigenetic age acceleration; IEAA: Intrinsic epigenetic age acceleration; MeFOSAA: 2-N-methyl-perfluorooctane sulfonamide acetate; n-PFOA: Linear perfluorooctanoate; n-PFOS: Linear perfluorooctane sulfonate; PFAS: Per- and poly-fluoroalkyl substance; PFDA: Perfluorodecanoate; PFHxS: Perfluorohexane sulfonate; PFNA: Perfluorononanoate; PFUnDA: Perfluoroundecanoate; Sb-PFOA: Sum of branched PFOA isomer; Sm-PFOS: Sum of perfluoromethylheptane sulfonate isomer; SE: Standard error.

Table 4. Significantly differentially methylated CpG sites by per- and poly-fluoroalkyl substances exposure among key hypothesis-driven genes ($p < 0.0025$).

PFASs with significant association	Probe ID	Gene name	Locus	Genic feature	Average DNA methylation at CpG site (%)	Effect estimate (SE)†	p-value
PFHxS	cg10457648	*NR1I2*	chr3:119497980	Near TSS	87.9	0.007 (0.002)	0.0018
	cg06491638	*HMGCS1*	chr5:43288815	3′ UTR	88.6	0.007 (0.002)	0.0014
	cg19699994	*ABCA1*	chr9:107554439	Gene body	84.9	0.017 (0.006)	0.0023
n-PFOA	cg23519812	*ACOT2*	chr14:74036744	Gene body; south shore	20.0	-0.026 (0.008)	0.0008
Sb-PFOA	cg21946299	*PPARG*	chr3:12329166	Near TSS; CpG island	2.9	0.005 (0.002)	0.0009
	cg20293950	*CD36*	chr7:80273190	5′ UTR	73.9	0.028 (0.009)	0.0017
PFNA	cg23236365	*ABCA1*	chr9:107618721	Gene body	83.4	0.012 (0.004)	0.0018
PFUnDA	cg13369760	*PPARG*	chr3:12446442	Gene body	93.9	-0.006 (0.001)	0.0000
	cg04029738	*FASN*	chr17:80049290	Gene body; CpG island	94.7	0.006 (0.002)	0.0024

†Effect estimates represent the proportion of methylation change per ln-transformed unit of PFASs (ng/ml for PFHxS, n-PFOA and PFNA) or the difference when comparing participants with detectable versus nondetectable concentrations (for categorical PFASs: Sb-PFOA and PFUnDA). Models adjust for age, gender, race (white vs all other), ethnicity (Hispanic and non-Hispanic), PC1, PC2, PC3, neutrophils, plasmablasts, $CD8^+$ naive T cells and $CD8^+CD28^-CD45RA^-$ cells. In total 433 CpG sites from 20 genes were selected based on differential expression after acute treatment to multiple PFASs in cultured human hepatocytes. Results are shown here for PFASs – DNA methylation relationships at loci with $p < 0.0025$ (0.05/20 genes).
chr: Chromosome; PFAS: Per- and poly-fluoroalkyl substance; PFHxS: Perfluorohexane sulfonate; PFNA: Perfluorononanoate; PFUnDA: Perfluoroundecanoate; n-PFOA: Linear perfluorooctanoate; Sb-PFOA: Sum of branched PFOA isomer; SE: Standard error; TSS: Transcription start site; UTR: Untranslated region.

Table 5. Significantly differentially methylated CpG sites by per- and poly-fluoroalkyl substances(at p < 9.4E-08).

PFASs with significant association	ProbeID	Gene Name	Locus	Genic feature†	Average % methylation at CpG Site	Effect estimate (SE)‡	p-value	q-value
Sb-PFOA	cg19385677	*CAPN12*	chr19:39233691	Gene body	75.8	0.020 (0.004)	4.29E-08	0.032
n-PFOS	cg19425289	*RAD1*	chr5:34917257	near TSS; south shore	75.6	-0.043 (0.007)	2.13E-08	0.016
PFDA	cg18210730	*TUBD1*	chr17:57970058	5′ UTR; CpG island	4.1	0.036 (0.006)	6.70E-09	0.005
	cg12435415	NA	chr3:186490344	North shore	0.8	0.013 (0.002)	6.43E-08	0.024
PFUnDA	cg01721356	*LOC339529*	chr1:244110987	Gene body	92.4	0.010 (0.002)	5.89E-09	0.004

†TSS and UTR; south/north shores and shelves are in relation to CpG islands. Model adjusted for: age, gender, race (white vs. all other), Hispanic ethnicity, PC1, PC2, PC3, neutrophils, plasmablasts, $CD8^+$ naive T cells and $CD8^+CD28^-CD45RA^-$ cells.
‡Effect estimates represent the proportion of methylation change per in-transformed unit of PFASs (ng/ml for n-PFOS and PFDA) or the difference when comparing participants with detectable PFASs to those with nondetectable concentrations (for categorical PFASs: Sb-PFOA and PFUnDA). Models adjust for age, gender, race (white vs all other), ethnicity (Hispanic and non-Hispanic), PC1, PC2, PC3, neutrophils, plasmablasts, $CD8^+$ naive T cells and $CD8^+CD28^-CD45RA^-$ cells.
chr: Chromosome; PFAS: Per- and poly-fluoroalkyl substance; PFUnDA: Perfluoroundecanoate; n-PFOA: Linear perfluorooctanoate; Sb-PFOA: Sum of branched PFOA isomer; TSS: Transcription start site; UTR: Untranslated region.

PFASs & DNA methylation: epigenome-wide approach

Four PFASs were associated with DNA methylation at specific loci using a strict p-value cutoff recommended for data from the EPIC ($p < 9e^{-8}$; Table 5). Sb-PFOA was positively associated with a CpG site within *CAPN12*, while n-PFOS was inversely associated with methylation near the transcription start site of *RAD1*. PFDA was positively associated with methylation in *TUBD1* and in a noncoding region. Participants with detectable PFUnDA concentrations had 1% higher methylation within a long noncoding RNA, *LOC339529* compared to the participants with nondetected PFUnDA. Using a relaxed q-value cutoff of 0.2, these four PFASs along with PFNA and MeFOSAA were associated with several more CpG sites (Supplementary Table 2). PFDA was associated with decreased methylation at 50 CpG sites and increased at 5 at $q \leq 0.2$, including in genes important for xenobiotic metabolism (*AHRR*), tumor cell progression or suppression (*CCL8, LOC642587, MIPOL1, PDIA4, RGS7, TNFAIP8L3* and *WWTR1* [81–87]), kidney disorders (*ANKS3* [88,89]), lipid metabolism (*SREBF1* [90]), immune activation and inflammatory response regulation (*DUSP19* and *GPD2* [91,92]) and reactive oxygen species production (*GPD2* and *PDIA4* [86,93]).

In the regional analysis considering consecutive CpG sites, the following PFASs were associated with DMRs: n-PFOS (1), PFNA (59) and PFDA (2; Supplementary Table 3). DMRs for n-PFOS and PFDA included genes encoding small nucleolar RNAs (snoRNAs). Among the many DMRs for PFNA, five were inversely associated with methylation in imprinted genes (*MEST, GNAS, PEG3, PEG10* and *PLAGL1*). PFNA was inversely associated with methylation at 15 consecutive CpG sites in the oncogene, *POU5F1* and 15 consecutive CpG sites in a gene involved in immune cell modulation, *SLFN12* [94]. PFNA was also associated with increased methylation in the pro-inflammatory cytokine, *IL32*; as well as in genes critical for regulating airway ciliary function (*TTLL1* [95]) and tumor suppression (*ZGPAT* [96]).

The biological pathways and functional gene sets that were enriched among the top differentially methylated genes for each PFASs included in this analysis by p-value are listed in Supplemental Table 4. n-PFOS results were enriched in eight gene sets, which included fatty acid oxidation, lipid transport and anion exchange. PFNA results were enriched in three gene sets, including for an immune related function (major histological complex class II protein complex). PFDA results were enriched in 23 gene sets that were primarily related to cell locomotion and migration and cation transport.

PFASs & blood cell composition

In adjusted linear regression, n-PFOS and PFDA were inversely associated with the estimated proportion of monocytes, and PFNA was inversely associated with NK cells ($p < 0.05$; Supplemental Table 5). Sm-PFOS, n-PFOS and PFNA were also inversely associated with $CD8^+$ T cells and/or $CD8^+$-naive cells in unadjusted models, but the associations were largely attenuated after adjusting for covariates.

Discussion

In this pilot cross-sectional study of structural firefighters in the USA, we report positive and significant associations between serum concentrations of some PFASs and epigenetic age biomarkers as well as DNA methylation at

specific loci and regions. Concentrations of PFHxS, n-PFOA and Sm-PFOS were associated with accelerated aging according to multiple epigenetic clocks. In the analyses to identify loci and regions associated with PFASs concentrations, n-PFOS, PFNA and PFDA were statistically significant predictors. Sb-PFOA and PFUnDA also had statistically significant associations with DNA methylation, but these PFASs were not widely detected in the study sample, and results should be interpreted carefully.

Our data suggest that several PFASs are correlated with accelerated epigenetic aging. Many studies have demonstrated significant correlations between accelerated epigenetic aging patterns and cancer, cardiovascular disease and other negative phenotypic outcomes [44–46]. Although all the mechanisms that drive these altered states of aging in tissue are not clear, it is evident that epigenetic age provides a more accurate assessment of biological age than chronological age [43]. Thus, individuals with an accelerated epigenetic aging pattern are likely at a higher risk for developing an age-associated phenotype or disease. Further, any individuals with an exposure that modifies one's epigenetic age (such as PFASs) may also be at a higher risk for early onset of age-associated diseases. Although not definitive or universal, such measures are helpful to understand the relative risks due to environmental exposures. A recent systematic review identified male sex, obesity, smoking, socioeconomic status and HIV infection as factors linked to accelerated epigenetic age [97], yet the impact of most chemical exposures on epigenetic age has rarely been studied to date. Thus, the association between PFASs and epigenetic age reported in this study merits further investigation as it is unknown whether other occupational exposures and conditions contributed to this association.

We were also interested in identifying genes and biological pathways with differential methylation associated with each PFAS. To this end, we began with a hypothesis-driven approach assessing DNA methylation at genes that had increased expression *in vitro* following exposure to three or more of the PFASs included in this study [70]. The *in vitro* study focused on genes relevant to cholesterol and lipid metabolism as dyslipidemia is one facet of PFASs toxicity. We report several genes with altered blood leukocyte DNA methylation in the present study (*ABCA1, ACOT2, CD36, FASN, HMGCS1, NR1I2* and *PPARG*). These same genes had increased expression following treatment with multiple PFASs in cultured hepatocytes. In addition, several of these genes have known roles in tumor function, especially those cancers that have a significant lipogenic phenotype. *ABCA1*, a multidrug membrane transporter, has key roles in cellular lipid and cholesterol homeostasis, HDL synthesis and tumor drug resistance, and it represents a potential tumor susceptibility marker [98,99]. *CD36*, a transmembrane receptor, possesses known roles in inflammation, angiogenesis, fatty acid metabolism, tumor metastasis and tumor-associated immune cells in the tumor microenvironment [100,101]. *FASN* contributes to many cancer cell processes including glycolysis, amino acid metabolism, proliferation, cytoskeleton remodeling, DNA repair and metastasis [102]. Recently, *HMGCS1* and its genetic variants were identified as kidney renal cell carcinoma and prostate cancer prognostic markers [103,104], respectively. Finally, *PPARG* possesses key roles in lipid metabolism, obesity and atherosclerosis with multiple known roles in numerous neoplasms and cancers, including prostate and thyroid [105–107]. Collectively, these genes should be evaluated further as potential biomarkers of effect for PFASs and/or risk for PFAS-induced disease. Furthermore, our comparative primary human hepatocyte *in vitro* model versus human blood samples displays possible utility of combining results from epidemiological and appropriate human *in vitro* studies to improve risk assessment efforts to identify hazardous exposures.

In the discovery approach using all CpG sites included on the EPIC array, n-PFOS, PFNA and PFDA had the most consistent evidence for differentially methylated sites, regions and pathways by exposure. Sb-PFOA and PFUnDA also had evidence for differential methylation, but these were not widely detected in the study sample; the results should be interpreted with caution. n-PFOS and Sb-PFOA had only a few statistically significant associations, and PFHxS had none. The statistically significant genes did not directly overlap to those reported in previous epidemiological studies of PFOA, PFOS and PFHxS with adult DNA methylation [54,55]. However, the previous studies had small sample sizes, reported higher PFASs concentrations, and these were performed in only men or only women. The associations with Sb-PFOA and n-PFOS reported in this study are in genes and pathways that could be relevant for toxicity. For example, *CAPN12*, *RAD1* and *DDR1* encode proteins that are involved in processes associated with carcinogenesis (e.g., regulation of cell death, cell growth and DNA repair [108–110]). In addition, several of the enriched pathways are involved in carcinogenesis (hippo signaling and lipid export from cells [111–113]) and lipid metabolism.

To our knowledge, this is one of the very few studies, and the first among adults, to identify differentially methylated genes associated with long-alkyl chain PFASs (PFNA, PFDA and PFUnDA); changes with important implications for immune system regulation and carcinogenesis. While these PFASs were not significantly associated with epigenetic clocks (with the exception of an inverse association with GrimAge), they were associated with the

greatest number of CpG sites, regions and pathways. In this study, we found that PFNA had associations with several critical genes involved in immune system regulation. PFNA was inversely associated with methylation at a region of the *SLFN12* gene, which has been shown to be upregulated during T-cell activation [94]. We also found a DMR encompassing the transcription start site of *IL32* that was positively associated with PFNA. The upregulation of *IL32* has previously been shown to be protective against viral infections [114]; thus an increase in *IL32* methylation may indicate decreased protection. Additional genes with differentially methylated sites or regions by PFNA have been implicated in cancers. Methylated *FNDC7* in circulating leukocytes has been suggested to have a role in smoking-induced lung cancer [115]. The GTPase *RAB37*, a vesicle trafficking regulator, is reported to play roles in immune cell function, gastric cancer [116] and nasopharyngeal metastasis [117]. Altered *PASK* expression is associated with aging [118], and is a prognostic survival marker due to its role in immune function in the stomach cancer microenvironment [119]. *NMBR* possesses key roles in testosterone secretion, proliferation and anti-apoptotic signaling in Leydig cells [117] and has a growth and angiogenesis role in lung cancer [120].

Many genes associated with PFDA in site-specific and regional analyses are also relevant to cancers and immune function. *RGS7*, a regulator of GPCRs, was found to be methylated in over 25% of renal cell carcinomas [121]. *ANKS3* expression is depleted in cystic kidney disease [122] and liver steatosis [123]. The ZNF protein, GLIS1, induces pluripotency in somatic cells via epigenetic mechanisms and is involved in several cancers including breast, leukemia, colon and thyroid [124,125]. *RAPGEF1* regulates proliferation, apoptosis, actin remodeling and migration in several cancers [126]. *MIPOL1* has tumor suppressor function [127]. *CCL8* is an important pro-inflammatory chemokine with known tumorigenicity roles and is a marker for poor prognosis for colorectal, pancreatic, renal, urothelial, testicular, breast and endometrial cancers [128]. *WWTR1*, a transcriptional coactivator, has known tumor enhancer activity, including vascular cancer [129]. Aryl hydrocarbon receptor repressor, *AHRR*, competes with *AHR* to suppress cell growth, differentiation and CYP p450 enzyme activity resulting in tumor suppression [130]. Increased DNA methylation at several loci in *AHRR* is a marker for smoking exposure, but here we report an inverse associated between PFDA and *AHRR* methylation in a different part of the gene. *PDIA4*, a key protein disulfide isomerase, shows increasing evidence for roles in tumor progression [86]. TIPEs, including *TNFAIP8L3*, possess key roles in inflammation, immune homeostasis and cancer development [131]. High *PCAT18*, a long noncoding RNA, was found to coincide with prostate cancer with potential roles in proliferation, apoptosis, migration and invasion abilities [132].

Epigenetic alterations by PFASs may be particularly relevant to risk for carcinogenesis. According to a recent review, epidemiological evidence is strongest, yet still sparse to date, for associations between PFASs and testicular and kidney cancer; there is also inconsistent evidence for an association with prostate cancer [133]. Studies consistently show that firefighters are at increased risk of incidence for specific cancer sites including of colon, prostate, testes, bladder and thyroid [134]. Epigenetic changes are one of the hallmarks of cancer and a key characteristic of a carcinogen [41] typically involving hypermethylation of tumor-suppressor genes and widespread hypomethylation of other regions of the genome [135,136]. The associations observed here (i.e., between PFASs serum concentrations and DNA methylation of cancer-related genes) represent the average across all cells in each sample for a given locus. A small effect size could mean a small change in all of the cells; alternatively it could represent a large change in a few cells or certain cell types within the sample. These changes can add up to key characteristics or initiating events in carcinogenesis or other toxic endpoints [41], and detecting them early before onset of overt disease has the ability to improve risk assessment of chemical exposures.

In addition to the cancer-related genes already described, several DMRs (for n-PFOS and PFDA) mapped to snoRNA genes. The main function of snoRNA is thought to be in posttranscriptional modification of rRNAs. However, emerging evidence links snoRNAs to tumorigenesis, and they may even be able to function as biomarkers for cancer diagnosis [137,138]. Interestingly, blood leukocyte methylation of a different snoRNA was associated with PFOS, PFOA and PFHxS exposure in a study of women with highly contaminated drinking water [54]. In the previous and current studies, PFASs were associated with increased methylation of these snoRNA genes in all cases.

There was limited evidence for associations between PFASs and estimates of cell type composition in the participants' blood samples (PFOS and PFDA with fewer monocytes; PFNA with fewer NK cells). However, these are from estimates based on DNA methylation data and not true counts. Regardless, drops in immunocyte number in blood can indicate potential accumulation in organs experiencing an immune response, while persistent alterations over long time scales indicate immunomodulation. Although lacking, recent evidence in rats points to PFASs exposure causing measurable changes in innate immune leukocyte populations. The National Toxicology Program found that PFOS exposure in male rats decreased blood leukocytes while both PFOS and perfluorobutane

sulfonate caused bone marrow hypocellularity [139]. This finding suggests PFASs exposure potentially impacts leukocyte development and differentiation processes. Conversely, NK cell populations were positively associated with PFHxS serum concentrations from exposed individuals in the C8 Study [140]. We observed similar positive associations between PFHxS and Sm-PFOS with estimated NK cells, although these were only statistically significant in the unadjusted model. A majority of PFASs immunotoxicity studies focus on immunosuppression associated with T-cell, B-cell and antibody production with little attention paid to effect of PFASs exposure on innate immune system function. Future research would benefit from evaluating PFASs exposures and perturbations of innate immune cell populations.

This study had several strengths. We quantified nine PFASs and employed a three-tiered approach for the epigenetic analysis including epigenetic age, a hypothesis-driven set of genes and an exploratory epigenome-wide analysis. This study also had limitations including the convenience nature of the population and cross-sectional study design. The pilot sample size was moderate to investigate relationships between PFASs concentrations and DNA methylation, and statistical power was limited to detect all true associations with small effect sizes. DNA methylation was quantified in blood leukocytes, yet epigenetic profiles differ by tissue type. Most study participants were male, white and non-Hispanic and these associations should be investigated in diverse populations. The PFASs panel measured in this population quantifies only legacy and long-alkyl chain PFASs and was developed based on community, not occupationally, exposed populations. We are likely missing other PFASs relevant to workers, including firefighters. PFASs concentrations represent the totality of exposures the firefighters experienced, and we do not know how much occupation versus environmental sources contributed to the measured concentrations. We assessed associations with each individual PFASs, however, future studies should explore the impact of the collective PFASs mixture.

Conclusion

In a pilot study of structural firefighters based in the USA, we report associations between PFHxS, n-PFOA and Sm-PFOS serum concentrations with accelerated epigenetic age. We also report associations between Sb-PFOA, n-PFOS, PFNA, PFDA and PFUnDA concentrations with DNA methylation of specific loci, and between n-PFOA, PFNA and PFDA with regions and gene sets. Statistically significant genes and gene sets have functions that include cancer-related pathways and immune function. Future work is needed to explore this in a larger sample size, with a broader range of exposure to PFASs and in a diverse population with regards to gender and race/ethnicity.

Future perspective

Epigenetic profiles of easily accessible samples such as blood leukocytes have the potential to serve as biomarkers of toxicity or to be developed into biomarkers that predict risk for future disease such as cancers. We are only beginning to understand the long term impacts of exposures to PFASs, a group of thousands of persistent chemicals. Harnessing epigenetic data from individuals occupationally or environmentally exposed to PFASs will improve understanding of cellular and molecular pathways underlying toxicity and disease risks that can be examined before clinical disease manifests. Such knowledge will be used to refine risk assessment of these hazardous exposures.

Summary points

- Firefighters are exposed to many occupational hazards including toxic and persistent chemicals such as per- and polyfluoroalkyl substances (PFASs).
- Epigenetic modifications, including DNA methylation, are responsive to environmental chemicals and are implicated in carcinogenesis.
- Accelerated epigenetic age, estimated from clocks based on DNA methylation data, is linked to many adverse health outcomes and overall mortality.
- We quantified serum concentrations of nine PFASs in structural firefighters and profiled blood leukocyte DNA methylation at >700,000 CpG sites via the Infinium MethylationEPIC array.
- Epigenetic data were also used to calculate seven markers of biological age, often referred to as 'epigenetic clocks', and three PFASs were associated with accelerated epigenetic age at two or more clocks each.
- Using a hypothesis-driven approach, we observed evidence for associations between PFASs and differential methylation at genes that had altered expression by multiple PFASs according to an *in vitro* study.
- Using an epigenome-wide approach, we observed significant associations between longer chain PFASs and DNA methylation at sites and regions in genes implicated in carcinogenesis, immune function, and other pathways.
- These findings build upon several other recent studies reporting associations between PFASs and epigenetics, and this potential biomarker of effect merits further consideration in risk assessment.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2021-0225

Author contributions

JL Burgess, MM Calkins, AJ Caban-Martinez, C Grant and JM Goodrich designed the research study; JL Burgess, AJ Caban-Martinez and MM Calkins obtained funding and designed protocols to develop the cohorts with consultation from C Grant, JM Goodrich, J Gulotta, D Wallentine, J Hughes and C Popp; J Gulotta, D Wallentine, J Hughes and C Popp recruited subjects and collected data; AM Jung, A Nematollahi , S Beitel, S Littau, MM Calkins and JL Burgess managed data and samples; AM Calafat and J Cook Botelho conducted exposure assessment; JM Goodrich, T Jenkins and A Dewald conducted the DNA methylation analysis; JM Goodrich conducted statistical analysis and led manuscript drafting with critical input from all coauthors and JM Graber, T Stueckle, AL Slitt, AM Jung and A Nematollahi provided interpretation of results and initial manuscript drafting. All authors contributed to the writing and/or editing of the manuscript and approve this final submission.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval from the University of Arizona IRB and the University of Miami IRB. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

Data requests will be reviewed by the Fire Fighter Cancer Cohort Study fire service Oversight and Planning Board to address firefighter concerns prior to determination of sharing de-identified data. This study is not a clinical trial.

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