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Serum Per- and Polyfluoroalkyl Substances and Urinary Phthalate Metabolite Concentrations in Relation to a Novel Marker for Immune Aging: A Cross-Sectional Study of U.S. Adults

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Serum Per- and Polyfluoroalkyl Substances and Urinary Phthalate Metabolite Concentrations in Relation to a Novel Marker for Immune Aging: A Cross-Sectional Study of U.S. Adults

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Abstract

Objective: Over 98% of US adults have detectable levels of per- and polyfluoroalkyl substances (PFAS), and nearly 75% have detectable phthalates, yet their immunological effects remain poorly understood. We examined mixture associations of phthalates and PFAS with immune aging in U.S. adults.

Methods: We analyzed two cycles of National Health and Nutrition Examination Survey data (2015-2018: n=2,479). Immune aging was defined as a novel four-category composite (normal, mild, moderate, severe) derived from absolute lymphocyte count, neutrophil-to-lymphocyte ratio, and high-sensitivity C-reactive protein. Confounders were identified via a directed acyclic graph. Advanced Bayesian Kernel Machine Regression (A-BKMR) evaluated mixture effects across five mixtures and two severity comparisons (mild and advanced [moderate/severe] immune aging). In both analyses, low molecular weight (LMW) and high molecular weight phthalate subgroups were created to account for their differing exposure pathways.

Results: Advanced immune aging was associated with age (prevalence risk ratio [PRR] 1.52, 95% credible interval [CrI]: 1.34, 1.73, comparing the 75th percentile with the median age), supporting the validity of the composite. Raising phthalates jointly from the 50th to the 75th percentile showed a weak positive trend with advanced immune aging (PRR: 1.08, 95% CrI: 0.98, 1.18). This trend was most apparent in males (PRR: 1.18, 95% CrI: 1.03, 1.34), though gender differences were not statistically distinguishable. In contrast, the same increase in the PFAS mixture was associated with lower risk (PRR: 0.85, 95% CrI: 0.77, 0.93). Mixtures were weakly associated with immune aging.

Conclusions: This exploratory composite, built from three routine blood tests, captures declining adaptive immunity and chronic inflammation and can be applied to existing biomonitoring data. Phthalates and PFAS showed divergent immune signatures. Rather than a protective effect, the inverse PFAS association may reflect immune suppression that single biomarkers can mask. The suggestive LMW phthalate signal, from chemicals common in fragrances and personal care products, warrants replication in longitudinal, survey-weighted cohorts.

Introduction

Per- and polyfluoroalkyl substances (PFAS) and phthalates are ubiquitous chemicals in our environment. The heat-stable carbon fluorine bonds in PFAS are commonly utilized in many industrial and everyday applications, such as building materials, or makeup.[1] On the other hand, phthalates are commonly used as plasticizers in industrial applications, and fixatives in personal care products.[2] These chemicals have been detected in multiple, representative cross-sections of the US population—PFAS (perfluorooctane sulfonic acid, perfluorooctanoic acid, etc.) in over 98%, and phthalates (metabolites of di-ethyl phthalate, di(2-ethylhexyl) phthalate) in about 75%.[3,4] Furthermore, PFAS and phthalates have been linked with adverse health outcomes, including endocrine and immune disorders.[2]

Specifically, phthalates have been associated with inflammation and oxidative stress in cross-sectional studies on Spanish samples including both male and females, using common biomarkers such as C-reactive protein, white blood cell count, and cytokines (e.g., Tumor Necrosis Factor-α, Interleukin-6, etc.).[5] Reviews have also identified PFAS to be associated with immune cell count.[6] However, to our knowledge, little is known on the association of PFAS and phthalates on immune aging—the degeneration, compensation, and reconstruction of the immune system with aging.[7] To our knowledge, current literature primarily considers adaptive immunity when quantifying immune aging (through T-cells), without accounting for innate immunity (e.g., neutrophils, monocytes, etc.).[8] The innate immune system serves as a first-responder, and activator of the adaptive immunity. Furthermore, an impaired adaptive immune response results in hyper-reactiveness of the innate response, causing chronic low-grade inflammation, called inflammaging. Thus, to understand immune aging as a whole, one must consider both the innate and adaptive immunity. Evaluating aging of the immune system is of particular importance as it compromises many attributes of one's health and well-being. As the immune system ages, shrinking of the thymus results in a much lower production of naïve T cells, thus impairing the adaptive immune response.[9] Similarly, innate immune function declines with age, as evidenced by reduced phagocytic capacity of macrophages[10], and impaired generation of pathogen-killing reactive oxygen species by neutrophils.[11] All these symptoms manifest in multiple physiological impairments such as a poor vaccine response, and increased susceptibility to chronic and acute infections[12]—outcomes especially harmful to older populations.

Our study addresses this gap in knowledge by examining the association of phthalates and PFAS with immune aging among US adults, utilizing two cycles of National Health and Nutrition Examination Survey (NHANES) data from 2015-2018. This study was reported according to STROBE guidelines.

Methods

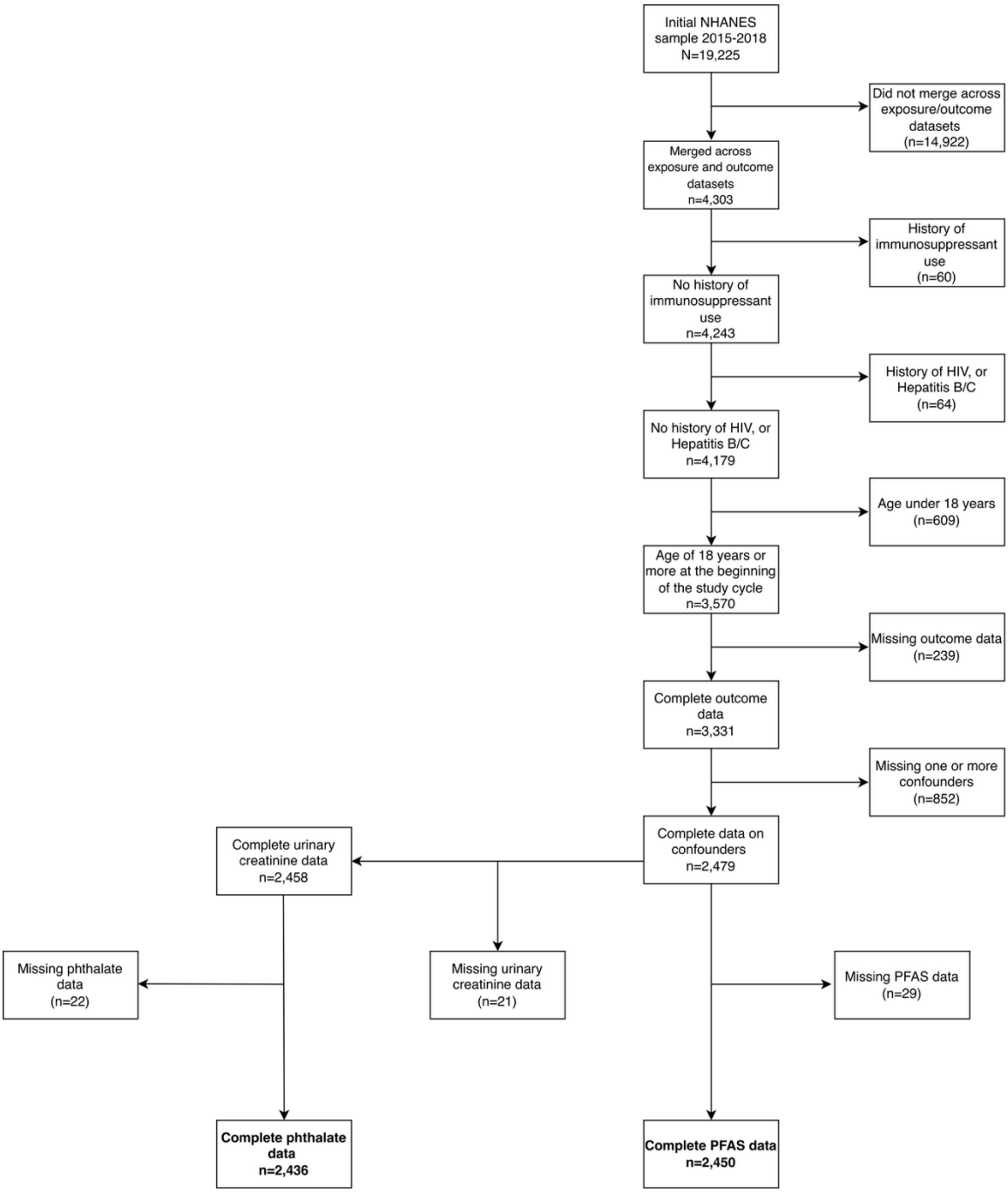
Study Population

The study had a serial cross-sectional design, by combining two cycles of NHANES data. NHANES utilizes a complex multi-stage design to select a nationally representative sample. This involves selecting Primary Sampling Units (PSUs), typically single counties, using the probability proportional to the size of the PSU. Typically, two individuals are chosen per household in each PSU.[13]

The analytic sample included all NHANES participants, aged 18 and over with matched exposure and outcome data. Individuals with a history of human immunodeficiency virus, hepatitis B, or hepatitis C, or with a history of immunosuppressant use were excluded to prevent participants with an already impaired immune system from skewing the outcome of interest. Next, individuals under the age of 18 were excluded from analysis, as adolescent immune function is different from that of adults.[14] Finally, observations with missingness for either exposure, outcome, or covariates, were excluded.

Across both NHANES cycles (2015-2018) we had 19,225 participants. After a series of exclusions for potential confounders and missing data, a total of 2,479 participants remained in the study. Of these, 2,450 participants were in the PFAS sample and 2,436 were in the phthalate sample. Sequential exclusions for each step are presented in Figure 1.

Figure 1. Flow chart for analytical sample.



Exposure

NHANES quantifies phthalate metabolite concentrations in urine samples,[15] whereas PFAS is measured in blood (serum) samples.[16] NHANES replaces any values below the given limit of detection (LOD) with $\frac{LOD}{\sqrt{2}}$. This substitution was put in place for accurate use of observations in analyses without counting exposure values as missing, and to aid in the calculation of geometric means.[17] Serum PFAS and urinary phthalate metabolite concentrations, with 75% or more of observations having values below the limit of detection, were excluded from this analysis, per Ferguson et al.[18] In total, 8 PFAS and 15 phthalates/phthalate-alternatives were included. Due to poor model convergence, only 18 of the total 23 biomarkers (12 phthalates, 6 PFAS) were analyzed, after an exclusion of variables with ≥40% of observations below the LOD.

To account for the differences in exposure pathways between low and high molecular weight phthalates, two classes of phthalates were created using their molecular weight. Phthalates with an ester side-chain of one to four carbons were placed in the low molecular weight (LMW) group, while phthalates with an ester side-chain of five or more carbons were placed in the high molecular weight (HMW) group.[19] The 12 measured phthalates by NHANES were classified as:

1. **Low-Molecular Weight (LMW) –** *Mono-ethyl phthalate, mono-n-butyl phthalate, mono-isobutyl phthalate, mono-3-hydroxy-n-butyl phthalate, Mono-2-hydroxy-iso-butyl phthalate, mono-(3-carboxypropyl) phthalate.* LMW phthalates have shorter alkyl chains and are less lipophilic and are primarily used for personal care/fragrance solvents.[19]
2. **High-Molecular Weight (HMW) –** *Mono(carboxynonyl) phthalate, Mono(carboxyoctyl) phthalate, Mono(2-ethyl-5-carboxypentyl) phthalate, Mono-(2-ethyl-5-hydroxyhexyl) phthalate, Mono-(2-ethyl-5-oxohexyl) phthalate, Mono-benzyl phthalate.* HMW phthalates have longer alkyl chains with more lipophilic properties (compared to LMW phthalates)—commonly used as plasticizers.[19]

Outcome

The outcome data were obtained from laboratory tests conducted at NHANES' mobile examination center. The outcome (immune aging) was constructed using two biomarkers: high-sensitivity CRP (hsCRP), and complete blood count (CBC). To assess hsCRP levels in a serum sample, hsCRP antibodies were introduced to a serum sample causing the proteins to aggregate via immunoturbidimetric methods.[20] Blood samples were also tested using a 5-part CBC differential to measure the levels of five types of white blood cells (neutrophils, lymphocytes, basophils, monocytes, eosinophils).[21]

An exploratory, four-category composite measure of immune aging was created using three biomarkers: absolute lymphocyte count, neutrophil-to-lymphocyte ratio (NLR), and hsCRP. Absolute lymphocyte count reflects adaptive immune function, with high levels potentially indicating active infection and low levels suggesting weakened immunity.[22] NLR compares neutrophil and lymphocyte counts and reflects the balance between innate and adaptive immune

responses. A higher NLR may indicate greater inflammation or reduced immune function.[23] hsCRP is an inflammatory marker with elevated levels indicating inflammation associated with infection, tissue injury, or chronic inflammatory conditions.[24]

Immune aging is characterized by two key features: reduced protective immunity and increased chronic inflammation.[12] To capture both features, each biomarker was dichotomized, with a value of 1 indicating a level consistent with reduced protective immunity or increased inflammation. Absolute lymphocyte count was coded as 1 for values below 1,500 cells/μL and 0 for values at or above 1,500 cells/μL.[25] Although 1,000 cells/μL is commonly used to define lymphopenia, a cut-off of 1,500 cells/μL was selected to capture less severe reductions in lymphocyte levels. NLR was coded as 1 for values above 4.0 and 0 for values at or below 4.0.[23] Similarly, hsCRP was coded as 1 for values above 3.0 mg/L and 0 for values at or below 3.0 mg/L.[26] The composite score ranged from 0 to 3 and was calculated by summing the three binary indicators, with higher scores indicating greater age-related immune dysfunction.

Covariates

Potential confounders were identified using a directed acyclic graph, which also provided the minimally sufficient adjustment set. The minimal set consisted of age, gender, socioeconomic status, body-mass index, race/ethnicity, smoking, alcohol consumption, and parity. Poverty-to-Income Ratio was derived using self-reported income and used as a proxy to assess socioeconomic status and serum cotinine levels were used as a proxy for smoking. Body-mass index was adjusted for as a continuous variable in all models. Differing hydration status can lead to differing levels of urinary phthalates.[27] Thus, urinary creatinine was included as a proxy for urine dilution, in the phthalate models. NHANES' recommended ethnicity variable, RIDRETH3, was included in the analysis because it disaggregates the Non-Hispanic Asian population, which was oversampled during the 2015-2018 cycles.[28] NHANES collects race/ethnicity data through self-reporting and was used as a covariate because of its known differences in PFAS/phthalate exposure and immune biomarkers across racial/ethnic groups in the US, which may reflect socioeconomic factors rather than biological differences. Alcohol consumption was defined as the self-reported average number of drinks consumed per drinking day in the past 12 months (0, 1-2, 3-4, 5 or more drinks). Parity was self-reported and controlled for in sensitivity analysis in female-only models to assess any potential residual confounding. The construction and detailed definitions for both alcohol and parity variables are explained in the supplement.

Statistical Analyses

We examined the distributions of continuous variables and summarized their central tendency and variability using means/medians and standard deviations/interquartile ranges, as appropriate. Significant differences in counts between the 2015-2016 and 2017-2018 cycle were tested using a chi-square test of homogeneity. Because the exposure distributions were skewed, Spearman correlations were estimated, and exposures were natural-log-transformed[29] for use as predictors in the models.

For the primary analyses, the advanced Bayesian kernel machine regression (A-BKMR) model was used with a probit link function. We fit two A-BKMR models—one compared the mild with the normal immune aging category, while for the second, the moderate and severe categories were collapsed into an advanced immune aging group and compared with the normal aging category. Thus, the two comparisons were mild versus normal immune aging and advanced versus normal immune aging. For each comparison, five exposure mixtures were examined: 1) all phthalates 2) six LMW phthalates, and 3) six HMW phthalates, 4) all 18 exposures, 5) all PFAS.

Models running the mild comparison ran 20,000 iterations, while different models running the advanced comparison ran different numbers of iterations, to maximize the effective sample size when running on a lower sample and to address initial convergence diagnostics. Matrix construction, scaling, knot selection, and model-fitting descriptions are detailed in the supplement and table S3.

The primary results obtained from A-BKMR were: 1) posterior inclusion probabilities (PIPs) for each exposure in each mixture, 2) overall mixture associations of the five mixtures on the two comparison groups, and 3) single predictor exposure-response curves for variables with PIPs ≥ 0.5. PIPs quantify the importance of a variable in its respective mixture[30], and we used the recommended threshold of 0.5 to identify major contributors.[31] The overall mixture effect was quantified using prevalence risk ratios (PRR) comparing the predicted probability of the outcome when all exposures in the mixture were set to a given percentile versus the median. This yields a risk ratio for each percentile in comparison to the median value, and a PRR was calculated for each percentile from 0.05 to 0.95, in intervals of 0.05. Single predictor exposure-response curves followed the same method, while changing the percentile of the exposure of interest, while holding all other exposures at their median.

To assess the association of chronological age with the immune composite, we used fitted A-BKMR models to estimate adjusted probabilities of mild and advanced immune aging at ages 33, 48, and 63 years, corresponding to the analytic sample's 25th, 50th, 75th age percentiles. PRRs comparing ages 33 and 63 years with age 48 years were calculated with each retained posterior draw and summarized using posterior means and 95% credible intervals (CrI). As the standard interaction term cannot be included in A-BKMR, we estimated posterior PRRs in male and female models separately while also standardizing to the same covariate distribution.[32] Differences in PRRs between males and females were estimated with 95% CrIs. Intervals excluding zero were interpreted as evidence of effect modification. For secondary and sensitivity analysis, the five mixtures were tested on the three biomarkers which made up the immune aging composite, and female-only models were run with and without parity to assess the potential residual confounding of parity. Additionally, we fitted the 10 primary models with additional adjustment for survey cycle given the differences observed in table 1. Stata version 17 and R version 4.5.3 was utilized for statistical analysis.

Results

The analytic sample comprised 2,479 participants, of which, 1,131 participants were from the 2015-2016 cycle, and 1,348 participants were from the 2017-2018 cycle. The proportion of participants across categories of serum cotinine, urinary creatinine, race/ethnicity, and alcohol consumption differed between the two cycles (serum cotinine: p = 0.02; urinary creatinine: p = 0.03; race/ethnicity: p = 0.001; alcohol consumption: p <0.001). Characteristics of the participants overall and by cycle are presented in Table 1.

About 51% of the overall participants had normal immune aging, while 42% had mild, 6% moderate and 1% had severe immune aging (Table 2). PFAS and phthalate metabolites showed right-skewed distributions. Observed concentrations varied by analyte (Table 3). For instance, the range of urinary mono-ethyl phthalate (MEP) concentrations observed in the analytic sample were between 0.9 ng/ml and 102,452 ng/ml, while the range of perfluorooctanoic acid was between 0.1 ng/mL and 52.8 ng/mL. Spearman correlations amongst phthalates (ρ: 0.18 to 0.97) and amongst PFAS were moderate to high (ρ: 0.12 to 0.85), while correlations between urinary phthalates and serum PFAS were low (ρ: -0.19 to 0.11, Figure S1).

In all models, age was positively associated with both advanced and mild immune aging (Table S1). Relative to the median age (48 years), the PRR at the 25th percentile (33 years) was 0.63 (95% CrI: 0.53, 0.73) for advanced and 0.92 (95% CrI: 0.88, 0.96) for mild immune aging, whereas for the 75th percentile (63 years), PRRs were 1.52 (95% CrI: 1.34, 1.73) and 1.08 (95% CrI: 1.04, 1.12), respectively.

Table 1. Characteristics of the study population in the two NHANES cycles, 2015-2016 and 2017-2018 (N=2,479).

	2015-2016		2017-2018		2015-2018		
Characteristic	**N (1,131)**	**%**	**N (1,348)**	**%**	**N(2,479)**	**%**	**P-value†**
Age							0.12
18-34	323	28.56	382	28.34	705	28.44	
35-44	193	17.06	190	14.09	383	15.45	
45-54	186	16.45	204	15.13	390	15.73	
55-64	189	16.71	258	19.14	447	18.03	
65 or older	240	21.22	314	23.29	554	22.35	
Poverty-Income Ratio Quintile							0.67
Most impoverished	235	20.78	274	20.33	509	20.53	
2	208	18.39	277	20.55	485	19.56	
3	228	20.16	271	20.10	499	20.13	
4	235	20.78	258	19.14	493	19.89	

Least impoverished	225	19.89	268	19.88	493	19.89	
Serum Cotinine*							0.02
<0.05 ng/mL (unexposed and non-smokers)	589	52.08	768	56.97	1,357	54.74	
0.05-3 ng/mL (second-hand smoke)	212	18.74	250	18.55	462	18.64	
>3 ng/mL (active smoker)	330	29.18	330	24.48	660	26.62	
Serum Cotinine Detection Rate*							0.11
At or above limit of detection	774	68.44	881	65.36	1,655	66.76	
Below limit of detection	357	31.56	467	34.64	824	33.24	
Urinary Creatinine**							0.03
Dilute (<30 mg/dL)	70	6.19	121	8.98	191	7.70	
Normal range (30 - 300 mg/dL)	1,013	89.57	1,166	86.50	2,179	87.90	
Concentrated (>300 mg/dL)	40	3.54	48	3.56	88	3.55	
Missing	8	0.71	13	0.96	21	0.85	
Urinary Creatinine Detection Rate**							0.28
At or above limit of detection	1,122	99.20	1,335	99.04	2,457	99.11	
Below limit of detection	1	0.09	0	0	1	0.04	
Missing	8	0.71	13	0.96	21	0.85	
Body Mass Index							0.73
Underweight (<18.5)	16	1.41	27	2.00	43	1.73	
Normal (18.5-25)	303	26.79	361	26.78	664	26.78	
Overweight (25-30)	348	30.77	408	30.27	756	30.50	
Obese (>30)	464	41.03	552	40.95	1,016	40.98	
Gender							0.35
Male	591	52.25	679	50.37	1,270	51.23	
Female	540	47.75	669	49.63	1,209	48.77	
Race/Hispanic Origin							0.001
Mexican American	189	16.71	187	13.87	376	15.17	
Other Hispanic	132	11.67	121	8.98	253	10.21	
Non-Hispanic White	420	37.14	513	38.06	933	37.64	
Non-Hispanic Black	249	22.02	284	21.07	533	21.50	
Non-Hispanic Asian	91	8.05	173	12.83	264	10.65	
Other Race or Multi-Racial	50	4.42	70	5.19	120	4.84	
Average number of drinks per drinking day in the past 12 months							<0.001
0 drinks	219	19.36	395	29.30	614	24.77	
1 to 2 drinks	579	51.19	641	47.55	1,220	49.21	

3 to 4 drinks	196	17.33	200	14.84	396	15.97	
5 or more drinks	137	12.11	112	8.31	249	10.04	
Parity							0.78
0 deliveries	110	9.73	136	10.09	246	9.92	
1	85	7.52	94	6.97	179	7.22	
2	140	12.38	147	10.91	287	11.58	
3	88	7.78	128	9.50	216	8.71	
4	56	4.95	73	5.42	129	5.20	
5 or more deliveries	52	4.60	64	4.75	116	4.68	
Not applicable	591	52.25	679	50.37	1,270	51.23	
Missing	9	0.80	27	2.00	36	1.45	

*LOD or Limit of detection for serum cotinine is 0.015 ng/mL
**LOD or Limit of detection for urinary creatinine is 5 mg/dL
†P-values were obtained using a chi square test for homogeneity, comparing counts across two survey cycles

Table 2. Frequency distributions of the immune aging composite in the PFAS (N = 2,450) and phthalate (N = 2,436) sample.

	PFAS sample		Phthalate sample		2015-2018	
Immune aging category	**N**	**%**	**N**	**%**	**N**	**%**
Normal	1,253	51.14	1,247	51.19	1,263	50.95
Mild	1,018	41.55	1,012	41.54	1,032	41.63
Moderate	146	5.96	145	5.95	151	6.09
Severe	33	1.35	32	1.31	33	1.33

Table 3. Characteristics of all exposures in NHANES 2015-2018 (ng/mL)

Class	Names	LOD	% > LOD	25th Percentile	50th Percentile	75th Percentile	Minimum	Maximum
PFAS (n=2,450)	PFDA	0.1	79.5	0.1	0.2	0.3	0.1	6.5
	PFHxS	0.1	98.9	0.7	1.2	2.0	0.1	28.7
	MeFOSAA	0.1	50.7	0.1	0.1	0.2	0.1	4.5
	PFNA	0.1	95.4	0.3	0.5	0.9	0.1	11.0
	PFUnDA	0.1	54.9	0.1	0.1	0.2	0.1	4.2
	n-PFOA	0.1	99.4	0.9	1.4	2.2	0.1	52.8
	n-PFOS	0.1	99.6	1.9	3.4	5.9	0.1	109.9
	Sm-PFOS	0.1	99.1	0.8	1.5	2.7	0.1	19.3
Phthalates & Phthalate-alternative metabolites (n=2,436)	MCNP	0.2	96.5	0.7	1.4	2.6	0.1	123.5
	MCOP	0.3	99.5	2.7	5.5	12.3	0.2	673.1
	MECP	0.4	99.7	4.2	8.2	14.9	0.3	458.0
	MHIBP	0.4	94.4	1.2	2.4	4.8	0.3	154.6
	MnBP	0.4	99.2	5.1	10.0	18.6	0.3	1792.3
	MCPP	0.4	79.5	0.5	1.0	2.0	0.3	294.5
	MCOCH	0.5	42.0	0.4	0.4	0.8	0.4	221.6
	MEP	1.2	99.7	13.0	31.2	85.6	0.9	102452.0
	MHBP	0.4	71.1	0.3	0.8	1.5	0.3	240.6
	MEHHP	0.4	99.0	2.6	5.2	9.9	0.3	144.7
	MHINCH	0.4	57.6	0.3	0.5	1.2	0.3	224.1
	MEHP	0.8	55.1	0.6	0.9	2.0	0.6	72.0
	MIBP	0.8	97.7	4.0	8.4	15.75	0.6	555.2
	MEOHP	0.2	99.1	1.6	3.4	6.3	0.1	141.1
	MBzP	0.3	96.0	1.4	3.4	8.3	0.2	307.2

Abbreviations:

MCNP - Mono(carboxynonyl) phthalate, MCOP - Mono(carboxyoctyl) phthalate, MECP Mono(2-ethyl-5-carboxypentyl) phthalate, MHIBP - Mono-2-hydroxy-iso-butyl phthalate, MnBP - Mono-n-butyl phthalate, MCPP - Mono-(3-carboxypropyl) phthalate, MCOCH - Cyclohexane-1,2-dicarboxylic acid, monocarboxyisooctyl ester, MEP - Mono-ethyl phthalate, MHBP - Mono-3-hydroxy-n-butyl phthalate, MEHHP - Mono-(2-ethyl-5-hydroxyhexyl) phthalate, MHINCH - Cyclohexane 1,2-dicarboxylic acid monohydroxy isononyl ester, MEHP - Mono-(2-ethyl)-hexyl phthalate, MIBP - Mono-isobutyl phthalate, MEOHP - Mono-(2-ethyl-5-oxohexyl) phthalate, MBzP - Mono-benzyl phthalate, PFDA - Perfluorodecanoic acid , PFHxS - Perfluorohexane sulfonic acid, MeFOSAA - 2-(N-Methyl-perfluorooctane sulfonamido) acetic acid, PFNA - Perfluorononanoic acid, PFUnDA - Perfluoroundecanoic acid, n-PFOA - n-perfluorooctanoic acid, n-PFOS - n-perfluorooctane sulfonic acid, Sm-PFOS - Perfluoromethylheptane sulfonic acid isomers

Advanced immune aging

In the A-BKMR analysis, phthalate mixture showed a modest positive relationship with advanced immune aging, in a dose-response manner (Figure 2a). Specifically, compared to setting all phthalates to their respective 50th percentile, setting all phthalates jointly to lower percentiles was associated with an PRR below 1, whereas setting them jointly to higher percentiles produced progressively higher PRRs. For the advanced immune aging comparison, the PRRs for the phthalate mixture were 0.90 (95% CrI: 0.79, 1.03) at the 25th percentile and 1.08 (95% CrI: 0.98, 1.18) at the 75th percentiles, relative to the median (Figure 2a). The exposure-outcome relationship of advanced immune aging with LMW phthalate mixture showed a similar pattern as the overall phthalate mixture (Figure 2b), whereas there was no association with HMW phthalates (Figure 2c). The PRRs for the PFAS mixture were 1.23 (95% CrI: 1.12, 1.34) at the 25th percentile, and 0.85 (95% CrI: 0.77, 0.93) at the 75th percentile, relative to the median (Figure 2e). The PRRs for the all-exposure mixture were largely similar to those for the PFAS mixture (Figure 2d), and the high group PIP for PFAS (0.99) indicated that PFAS contributed substantially to the overall mixture association.

Although the CrIs for the difference in PRRs between males and females included 0 for all mixtures (Figure 3), there was a signal for a positive association in males, but not in females (Figure 4). In males, PRRs for phthalates were 0.84 (95% CrI: 0.71, 0.99) at the 25th percentile and 1.18 (95% CrI: 1.03, 1.34) at the 75th percentile, relative to the median (Figure 4c). A similar relationship was observed for LMW phthalates (Figure 4e) but not for HMW phthalates (Figure 4g). In females, opposite trends were observed for all three phthalate mixtures (Figure 4d, 4f, 4h). PFAS and all-exposure mixtures showed negative associations in both genders (Figure 4a, 4i, 4b, 4j).

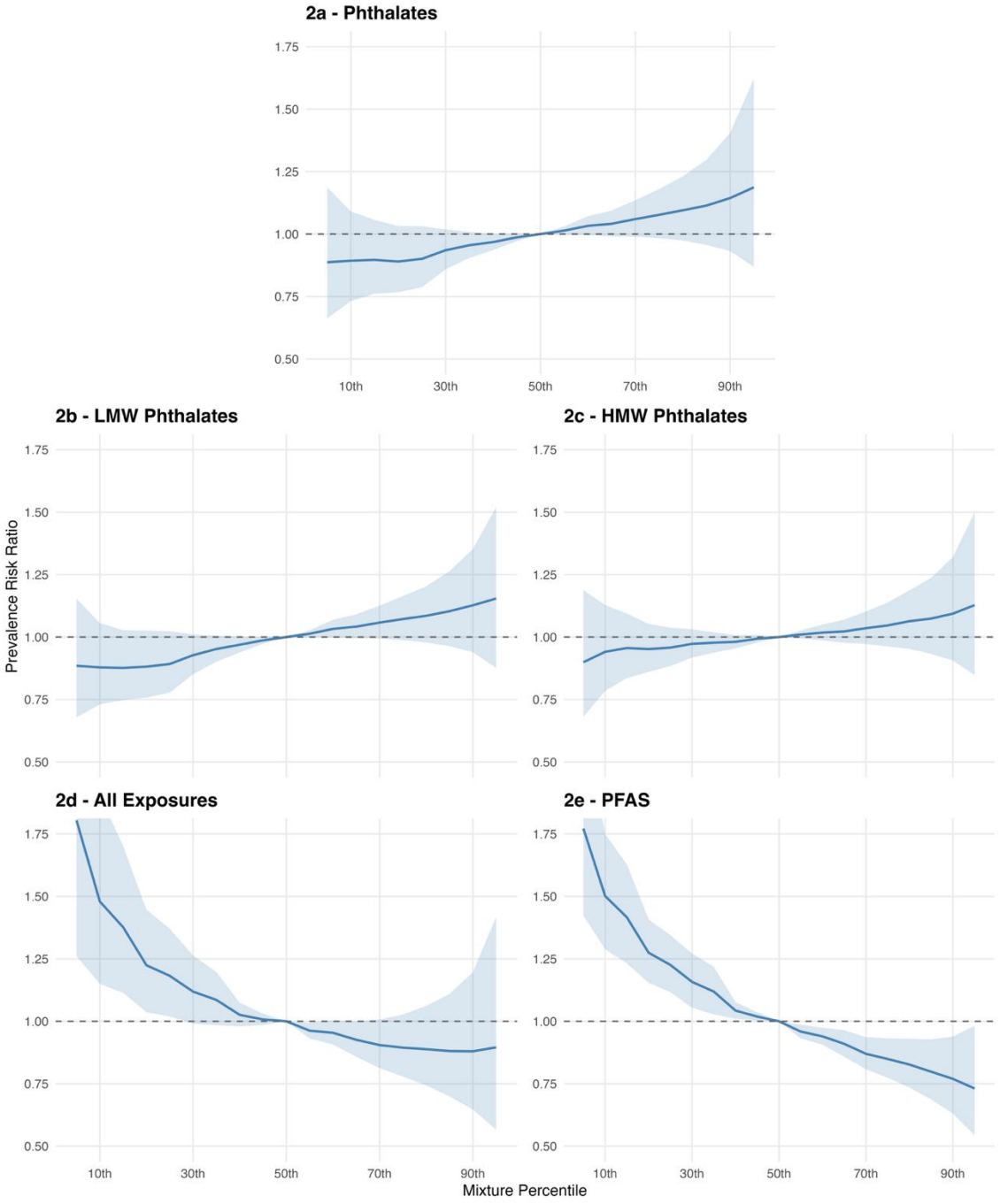
Mild immune aging

None of the five mixtures were associated with mild immune aging, nor were there any difference in association by gender (Figure S5, S8, S9). Perfluorooctanoic acid (PFOA) was the only exposure with a PIP>0.5, specifically for the PFAS and all-exposure mixture for the mild immune aging comparison (Table S2a). The single-predictor exposure-response curve showed a similar negative association to that of the PFAS and all-exposure mixture in the mild immune aging comparison (Figure S10).

Sensitivity Analyses

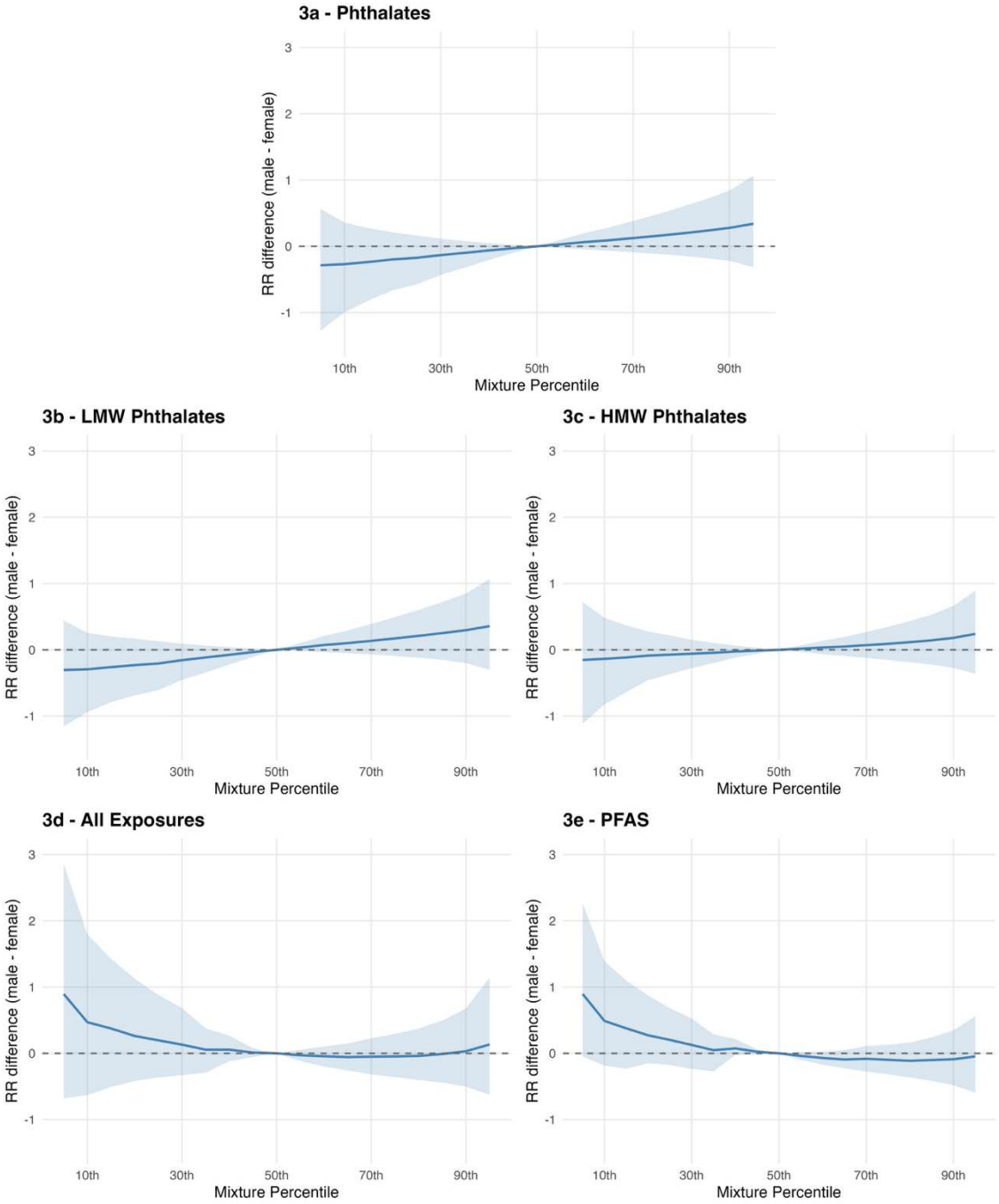
In sensitivity analyses, additional adjustment of the female-only models with parity did not change the results (Figures 4, S6, S7, S8). Likewise, additional adjustment of the survey cycle in primary models did not change the observed results for both advanced and mild immune aging (Figure S11, S12). Among the three continuous immune biomarkers analyzed as secondary outcomes, the phthalate and LMW phthalate mixtures showed a weak U-shaped pattern with NLR (Figure S3a, S3b), while the PFAS mixture showed a monotonic inverse association with NLR across the exposure range (Figure S3e). No associations were observed with absolute lymphocyte count or hsCRP (Figure S2, S4).

Figure 2. Overall associations of mixtures in Advanced Bayesian Kernel Machine Regression, in the advanced compared to normal immune aging group.



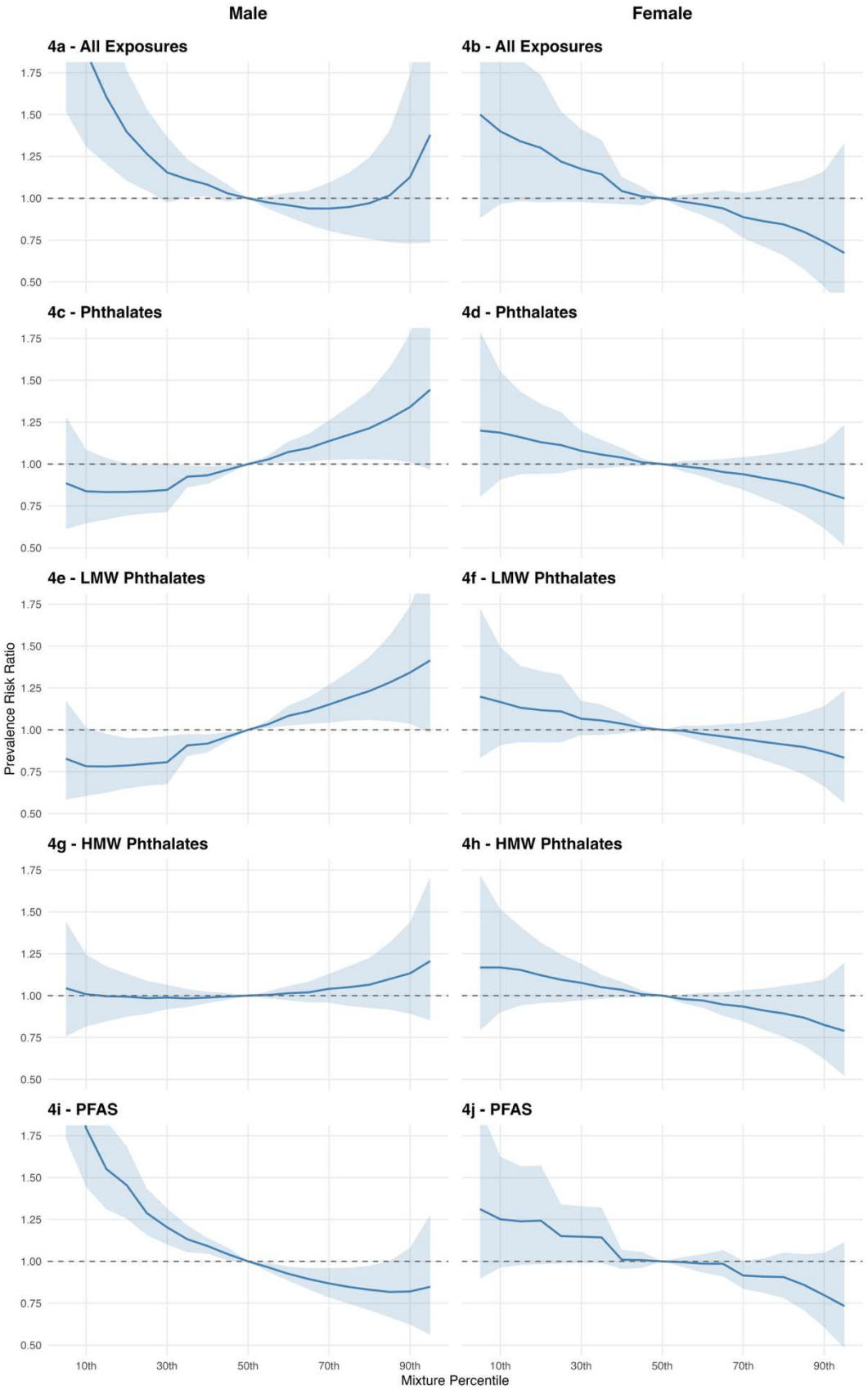
Note: Mixture associations were obtained using the median as the reference, and the shaded region represents the 95% credible interval

Figure 3. Posterior risk ratio differences of the overall mixture association, comparing male-and female-only models.



Note: Mixture associations were obtained using the median as the reference, and the shaded region represents the 95% credible interval

Figure 4. Overall associations of mixtures in Advanced Bayesian Kernel Machine Regression, in the advanced compared to normal immune aging group, for models stratified by gender.



Note: Mixture associations were obtained using the median as the reference, and shaded regions represent the 95% credible interval

Discussion

In this analysis of two cycles of NHANES data (2015-2018) we examined serum PFAS and urinary phthalate metabolite mixtures in relation to immune aging using a novel composite that captures both reduced adaptive immunity and chronic inflammation. Associations were largely confined to advanced immune aging. Phthalates showed a modest positive trend, more apparent for LMW phthalates, and most pronounced in males, though male-female differences were not statistically distinguishable. Higher PFAS exposure was negatively associated with advanced immune aging, and the all-exposure mixture followed the same pattern, consistent with PFAS contributing more to the mixture than the phthalates. Together, these findings suggest that PFAS and phthalates may be associated with divergent immune profiles.

Corroborating this study's findings, prior literature conducted in humans showed associations between exposure to urinary phthalates, such as mono-benzyl phthalate, with C-reactive protein in an NHANES sample of 10,031 individuals (1999-2006).[18] More recent studies have shown positive associations between urinary phthalate exposure and expression of pro-inflammatory cytokines, such as interleukin(IL)-18, IL-12, IL-8, and IL-1β in Chinese samples.[33] During an inflammatory response, these cytokines recruit, activate, and direct immune cells to sites of inflammation. Specifically, IL-1β triggers elevated secretion of C-reactive protein,[34] IL-8 is responsible for an influx of neutrophils and other innate immune cells,[35] and IL-12 is necessary for the maturation of naïve T cells for an adaptive immune response.[36] Therefore, we hypothesize an elevated hsCRP and NLR may be a consequence of an elevated IL-1β, and IL-8, consistent with our findings of LMW phthalates and NLR in continuous A-BKMR models. Although the CrIs for male-female differences included 0, the positive phthalate association in males but not females, who showed opposite trends, may reflect gender differences in exposure patterns. We consider the stratified analysis as exploratory and warrants replication in larger samples.

The negative association of PFAS on immune aging is intriguing, which may reflect immunomodulation rather than protection. Because the outcome was defined by markers of lymphopenia and systemic inflammation (low lymphocyte count, elevated NLR, elevated hsCRP), a negative association does not necessarily indicate protection against immune aging. One possible explanation is that PFAS lower common biomarkers of immune aging (i.e., inflammation) without reflecting healthier immune function[37] though our data cannot test this, as the composite may not capture such pathways. For example, PFAS have been shown to mimic fatty acids with their carbon-fluorine bonds, which may enable them to bind to the ligand binding domains of the peroxisome proliferator activated receptor (PPAR) thereby activating them.[38] Activated PPARs exert inhibitory effects on transcription factors such as nuclear factor kappa B, thus acting as anti-inflammatory receptors. However, bioaccumulation of PFAS, coupled with their persistent half-lives, could cause sustained activation of PPAR receptors, and a prolonged anti-inflammatory state. Our findings are directionally consistent with prior studies reporting positive associations between PFAS and lymphocyte counts, and negative associations with neutrophils.[39]

PFAS and phthalate exposure vary across populations and sampling periods, limiting direct comparisons between countries. International birth-cohort comparisons demonstrate substantial variation in maternal PFAS concentrations and highlight the importance of sampling year when interpreting geographic differences.[40] In our analytic sample, median serum n-PFOA was 1.4 ng/mL, lower than the geometric mean total PFOA concentrations of 4.7 ng/mL among American Red Cross donors sampled in 2000-2001 and 3.9 ng/mL in NHANES 2003-2004. These comparisons are consistent with declining U.S. PFOA exposure, although differences in population composition, summary measures, and isomer coverage preclude direct equivalence.[4,41] Phthalate exposure patterns likewise differ by compound and population, as a multicountry biomonitoring study identified mono-ethyl phthalate as the predominant urinary metabolite in samples from India and Kuwait, whereas other metabolites predominated in China, Korea, and Vietnam.[42] Our median urinary mono-ethyl phthalate concentration of 31.2 ng/mL was below the 391 ng/mL reported in that study's Kuwait sample, illustrating the broad variation in measured exposure across countries. Collectively, these comparisons place our findings within a specific U.S. exposure period, and emphasize the need for caution during comparisons of mixture associations across different populations with different exposure concentrations and compositions.

The primary limitation of this project is its serial cross-sectional study design. Cross-sectional collection of all data prevents the establishment of temporality preventing any causal inference, particularly for chemicals with short half-lives (i.e., phthalates, and new PFAS). Legacy PFAS (PFOS, PFOA, etc.) have half-lives on the scale of multiple years, allowing for single-point biomonitoring to provide a more reliable measure of long-term exposure. Second, the immune aging composite incorporated lymphocytes twice, through absolute lymphocyte count and NLR, so lymphocyte levels may contribute disproportionately to the composite. In addition, the cutoff of 1,500 cells/μL for absolute lymphocyte count is less established than the NLR (>4.0) and hsCRP (>3.0 mg/L) cutoffs, and the composite has not been validated against clinical outcomes, allowing for the possibility of outcome misclassification, though the composite was associated with chronological age. Additionally, the use of datasets from 2015-2018 may not reflect current exposure profiles, due to ongoing regulations of these chemicals in the US. Particularly, regulations such as EPA's 2024 National Primary Drinking Water Regulation for PFAS under the Safe Drinking Water Act[43] and the Prohibition of Children's Toys and Child Care Articles Containing Specified Phthalates of 2018[44] have likely altered environmental and consumer exposure levels. Next, race/ethnicity was self-reported using NHANES' broad, pre-defined categories. As a social construct, this was included as a proxy for unmeasured structural/socioeconomic factors rather than any assumed biological basis for the observed associations. Additionally, results may not be generalizable to the greater US population, as A-BKMR's current model configuration prevents the usage of survey weighting in its Markov Chain Monte Carlo. NHANES' survey weights ensure that sample findings are generalizable to the general, non-institutionalized US population.[45] Finally, NHANES' measurement of phthalate exposure via urinary metabolite causes exposure data to represent short-term exposure windows, rather than an individual's exposure patterns, which could likely be chronic given the ubiquitous presence of PFAS and phthalates in daily use products.

This study addresses several gaps in the existing literature. Most of the current literature researching PFAS and phthalates focuses on specific organ systems, such as endocrinology, or

reproductive health.[46] Prior research examining PFAS and phthalates in relation to immune function has predominantly focused on individual biomarkers, such as C-reactive protein or gamma-glutamyl transferase.[47] In addition to these biomarkers, white blood cell count is also used to approximate immune function.[39] The novel composite uses a combination of these biomarkers (i.e., C-reactive protein, absolute lymphocyte count) to model both innate and adaptive immunity. As a check on composite validity, the composite was positively associated with chronological age. These biomarkers are commonly found in datasets like NHANES, making this composite design applicable to other studies. Additionally, the use of two pooled NHANES cycles increases statistical power relative to single-cycle analysis. Finally, the application of A-BKMR to five distinct mixture configurations—including the LMW and HMW subgroups—allowed for flexible estimation of mixture associations that single-pollutant models cannot capture with highly correlated predictors.

In summary, in a national sample of U.S. adults with biomonitored exposures, serum PFAS and urinary phthalate mixtures showed divergent associations with a composite of immune aging capturing both reduced adaptive immunity and chronic inflammation. These findings have three implications for exposure assessment and regulation. First, the inverse association of PFAS with advanced immune aging, should not be interpreted as protective, as this is more consistent with PFAS altering immune function through pathways that single biomarkers or composites can mask[38] and it argues for multi-marker immune endpoints in PFAS risk assessment rather than reliance on any one immune biomarker. Additionally, the routine collection of lymphocyte levels, NLR, and hsCRP levels in NHANES allows this composite to be applied directly to existing biomonitoring data to track immune health alongside chemical exposure. Finally, the suggestive phthalate association of LMW phthalates with advanced immune aging, particularly in males, is relevant to the use of these phthalates in fragrances and personal care products, which are not covered by current U.S. restrictions on phthalates.[48] If replicated in longitudinal cohorts, these results would support extending exposure-reduction efforts, such as ingredient disclosure and source reduction, to LMW phthalates, and including immune aging as an endpoint in cumulative risk assessment of PFAS and phthalates.

Supporting Information

The supplement contains further detail regarding NHANES design, covariate definitions, and specifications regarding A-BKMR analyses. The supplemental information also includes Tables S1-S6, and Figures S1-S12.

Ethics Statement

The NHANES protocol was approved by the National Center for Health Statistics Research Ethics Review Board. All participants provided written informed consent. The current analysis used publicly available, de-identified data and was therefore exempt from additional IRB review.[49]

Data Availability

All data for this study was obtained from the National Center for Health Statistics', National Health and Nutrition Examination Survey. Data files can be obtained on the public website of NHANES, and a table of all datasets used for this study can be found in table S6.

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Conflict of Interest Statement

The authors declare they have no actual or potential competing financial interests.

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