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DOI: 10.1111/all.13669

Cord-serum per- and poly-fluoroalkyl substances and atopy and eczema at 12-months

To the Editor,

The rapid rise in allergic disease prevalence associated with "Westernization" indicates that environmental exposures are likely to be critical for the development of allergic diseases. As allergic diseases often occur very early in life, in utero and early life exposures while the immune system is still maturing are likely to be important. Exposures to per- and poly-fluoroalkyl substances (PFAS) have received increasing attention.¹ PFAS were adopted into manufacturing processes in the 1950s due to their surfactant and surface chemical properties and were rapidly introduced into a wide range of products including food packaging. Concern about potential health impacts of these chemicals continues to increase as they have become ubiquitous in the environment. Some PFAS, such as perfluorooctane sulphonic acid (PFOS), bio-accumulate as they bind to lipoproteins in blood and are neither metabolised nor effectively excreted from the body as parent compounds. While a number of these chemicals have been phased out of manufacturing, other PFAS have been introduced to replace them.

Human exposure to PFAS is almost universal with global serum samples yielding measurable amounts, and there is evidence these compounds may induce immune suppression.² Serum levels of PFAS in general population samples appear to have peaked during the 1990s, with levels of many PFAS having subsequently declined.³ However, most cord blood samples continue to contain detectable levels of PFAS,⁴ indicating ongoing maternal-infant exposure which may affect immune development during this critical time period. Very few studies have assessed the association between early life PFAS exposure and allergic disease outcomes,⁵⁻⁸ and these have shown mixed results.

To determine if PFAS in cord blood samples were associated with risks of eczema and allergic sensitisation, the earliest markers of allergic disease, we nested a case-control study within the Melbourne Atopy Cohort study.⁹ We selected 60 children with stored cord-serum samples (see Table S1 for demographic details) based on their sensitisation and eczema status at 12 months of age: 15 each of: (a) neither; (b) atopy only (no eczema); (c) non-atopic-eczema (eczema but no sensitisation); and (d) atopic-eczema (both eczema and sensitisation). Individual sensitisation

was assessed using skin prick tests to six allergens and atopy was defined as a 2 mm or greater wheal to one or more allergens. Eczema was defined by either a parent report of a doctor diagnosis of "eczema" or use of topical steroids for a rash excluding the nappy area.⁹ A total of 23 PFAS were assessed using liquid chromatography coupled with tandem mass spectrometry (see Data S1 for further details). Associations between levels of PFAS and the outcomes of eczema and atopy were assessed using multinomial logistic regression models. Adjustments were made for potential confounding factors, including sex and maternal education.

Of the assessed PFAS, 10 were detected in <5% of participants and excluded from further analyses (Table S2). Values were dichotomized (detected vs not) for the six PFAS that were detectable in 10-95% of samples. The remaining seven PFAS were treated as continuous exposures with any non-detected samples allocated a value of zero. The level of each PFAS correlated at most moderately (highest correlation = 0.53) with other measured PFAS (Table S3).

The majority of assessed PFAS tended to be associated with reduced risks of sensitisation or eczema (Table 1). These associations were significant for perfluoroundecanoic acid (PFUnDA) (all outcomes) and N-methyl perfluoro-1-octanesulfonamidoacetic acid (NMeFOSAA) (non-atopic-eczema and atopic-eczema). Most of these associations were not materially changed when adjusted for parent characteristics and child sex (Table 2 and Figure S1). However, perfluorohexanoic acid (PFHxA) became significantly associated with atopy, and N-ethyl perfluoro-1-octanesulfonamidoacetic acid (NEtFOSAA) became associated with atopic-eczema (there was evidence of confounding by maternal age and education in both cases). When associations were further adjusted for the other significant PFAS, PFUnDA remained associated with all outcomes, NEtFOSAA was associated with atopic eczema, while associations were no longer detected for NMeFOSAA (data not shown).

Our results are consistent with the few studies that have been published in this area. Like Wang et al,⁸ who assessed associations in 244 children from Taiwan, we did not see associations between cord serum levels of PFOA, PFOS or perfluorononanoic acid (PFNA) and eczema. Unfortunately, Wang et al did not measure the PFAS for which we observed associations.

TABLE 1 Unadjusted associations between per- and poly-fluoroalkyl substances (PFAS) and eczema and atopy outcomes at 1 y of age

PFAS	Acronym	Atopy (no eczema) ^b mOR (95% CI)	Non-atopic eczema ^b mOR (95% CI)	Atopic eczema ^b mOR (95% CI)
Linear effect (per 1 standard deviation increase)				
Perfluorooctane sulphonic acid	PFOS	0.56 (0.26-1.23)	0.78 (0.39-1.57)	0.72 (0.35-1.47)
Perfluorooctanoic acid	PFOA	1.05 (0.52-2.12)	1.08 (0.54-2.19)	0.66 (0.30-1.45)
Perfluorohexane sulfonic acid	PFHxS	0.66 (0.30-1.46)	0.76 (0.38-1.53)	0.80 (0.41-1.56)
Perfluoroundecanoic acid	PFUnDA ^c	0.04 (0.01-0.20)	0.04 (0.01-0.22)	0.10 (0.02-0.46)
Perfluorobutanesulphonic acid	PFBS	1.14 (0.55-2.38)	1.24 (0.60-2.54)	1.07 (0.50-2.25)
Perfluorononanoic acid	PFNA	0.72 (0.35-1.49)	0.68 (0.32-1.44)	0.42 (0.17-1.06)
N-ethyl perfluoro-1-octanesulfonamidoacetic acid	NEtFOSAA ^c	0.89 (0.47-1.69)	0.45 (0.19-1.10)	0.54 (0.24-1.21)
Any vs not detected				
N-methyl perfluoro-1-octanesulfonamidoacetic acid (40/60)	NMeFOSAA ^c	0.14 (0.01-1.42)	0.08 (0.01-0.79)	0.08 (0.01-0.79)
Perfluoroheptanoic acid (23/60)	PFHpA	4.00 (0.88-18.26)	0.50 (0.10-2.63)	1.00 (0.22-4.56)
Perfluorodecanoic acid (21/60)	PFDA	0.42 (0.09-1.92)	0.57 (0.13-2.50)	0.57 (0.13-2.50)
N-ethyl perfluoro-1-octanesulfonamido-ethanol (14/60)	NEtFOSE	0.55 (0.12-2.55)	0.23 (0.04-1.41)	0.11 (0.01-1.04)
Perfluorohexanoic acid (9/60)	PFHxA	0.42 (0.06-2.77)	0.69 (0.12-3.79)	^a NE
Perfluorooctanesulfonamide (7/60)	FOSA	0.29 (0.03-3.12)	^a NE	1.00 (0.17-5.99)

^aNot estimable. No child with atopic eczema had detectable levels of PFHxA (exact $P = 0.10$). No child with non-atopic eczema had detectable levels of FOSA (exact $P = 0.22$).

^bReference category was children without eczema or atopy. Associations expressed as multinomial odds ratios (mOR) with their 95% confidence intervals (95% CI). Associations with $P < 0.05$ are in bold.

^cSecond transitions for a subset of samples could not be confirmed as levels were close to the limit of quantification.

TABLE 2 Adjusted^c associations between per- and poly-fluoroalkyl substances (PFAS) and eczema and atopy outcomes at 1 y of age

PFAS	Acronym	Atopy (no eczema) ^a mOR (95% CI)	Non-atopic eczema ^a mOR (95% CI)	Atopic eczema ^a mOR (95% CI)
Linear effect (per 1 standard deviation increase)				
Perfluorooctane sulfonic acid	PFOS	0.38 (0.13-1.16)	0.68 (0.30-1.52)	0.64 (0.28-1.46)
Perfluorooctanoic acid	PFOA	0.69 (0.25-1.89)	0.67 (0.26-1.69)	0.29 (0.09-1.01)
Perfluorohexane sulfonic acid	PFHxS	0.81 (0.29-2.29)	0.79 (0.36-1.73)	0.88 (0.40-1.93)
Perfluoroundecanoic acid	PFUnDA ^b	0.01 (<0.01-0.15)	0.01 (<0.01-0.18)	0.03 (<0.01-0.52)
Perfluorobutanesulfonic acid	PFBS	1.56 (0.57-4.29)	1.53 (0.68-3.46)	0.93 (0.35-2.46)
Perfluorononanoic acid	PFNA	0.66 (0.26-1.72)	0.64 (0.29-1.44)	0.33 (0.09-1.17)
N-ethyl perfluoro-1-octanesulfonamidoacetic acid	NEtFOSAA ^b	0.87 (0.31-2.47)	0.29 (0.07-1.12)	0.11 (0.02-0.60)
Any vs not detected				
N-methyl perfluoro-1-octanesulfonamidoacetic acid (40/60)	NMeFOSAA ^b	0.13 (0.01-2.05)	0.05 (<0.01-0.61)	0.06 (0.01-0.76)
Perfluoroheptanoic acid (23/60)	PFHpA	4.37 (0.61-31.05)	0.30 (0.04-2.51)	0.68 (0.10-4.71)
Perfluorodecanoic acid (21/60)	PFDA	0.59 (0.09-3.80)	0.69 (0.13-3.70)	0.41 (0.07-2.29)
N-ethylperfluoro-1-octanesulfonamido-ethanol (14/60)	NEt FOSE	0.46 (0.06-3.80)	0.09 (0.01-1.01)	0.09 (0.01-1.36)
Perfluorohexanoic acid (9/60)	PFHxA	0.01 (<0.01-0.52)	0.48 (0.05-4.78)	^d NE
Perfluorooctanesulfonamide (7/60)	FOSA	0.59 (0.04-9.55)	^d NE	0.44 (0.05-4.07)

^aReference category was children without eczema or atopy. Associations expressed as multinomial odds ratios (mOR) with their 95% confidence intervals (95% CI). Associations with $P < 0.05$ are in bold.

^bSecond transitions for a subset of samples could not be confirmed as levels were close to the limit of quantification.

^cAll associations adjusted for sex, parity (first vs subsequent born infant), parental age and history of eczema and smoking, and mother's education.

^dNE- not estimable. No child with atopic eczema had detectable levels of PFHxA. No child with non-atopic eczema had detectable levels of FOSA.

A potential association between early life PFUnDA exposure and reduced risk of eczema was reported by Okada et al,⁶ who examined associations between late pregnancy maternal plasma PFAS levels

and allergic disease outcomes up to 2 years in ~2000 mother/infant pairs from Japan. They observed that among girls, when compared with infants of mothers in the lowest quartile of PFUnDA

concentration, infants of mothers in the highest quartile had a reduced risk of eczema. This association was not seen in boys. In our own study, we also observed a somewhat stronger association between PFUnDA and eczema in girls (OR = 0.18, 0.04-0.73) than boys (P-interaction = 0.14). Okada et al⁶ also observed associations between perfluoro-n-tridecanoic acid (PFTriDA) and eczema, but no participant in our cohort had detectable levels of this PFAS.

The only other study published to date on this topic examined maternal serum during pregnancy in children from Ukraine and Greenland (n = 1024) and found no association between PFUnDA and ever-eczema (0.95, 0.79-1.15) in 5-9-year-olds, which is much older than the present study.⁵ Associations between early life NtFOSAA and NMeFOSAA exposure and allergic disease outcomes have not been previously reported.

At this time, we are not able to exclude the possibility of unmeasured confounding or ongoing exposure beyond the intrauterine period. We were also not able to examine associations with long term and other allergic diseases outcomes. We are currently seeking to measure these PFAS in all our stored samples.

The results of this small nested case-control study of children born between 1990 and 1994 suggest that early life PFAS exposure may impact on eczema and sensitisation. Interestingly, a protective effect was observed for PFUnDA, a result mirrored in a Japanese cohort,⁶ possibly suggesting that it may be causing immune suppression. Furthermore, there is a growing body of evidence of possible immune suppression caused by PFAS in early life,² making this an area requiring urgent investigation in larger studies. However, given the multiple comparisons made in this study, the result should be interpreted with caution at this time.

ACKNOWLEDGMENTS

We thank Christine Axelrad (Murdoch Children's Research Institute, Melbourne, Australia), David Hill (Royal Children's Hospital), Cliff Hosking (John Hunter Children's Hospital, Newcastle, Australia) and John Thorburn (Mercy Maternity Hospital, Melbourne, Australia) for their contributions in the early phase of the study. We thank Anne Balloch for assistance with data management, and Helen Tsimiklis and Mark Fernando for bio-specimen management. Most importantly, we thank all of the MACS children and parents for their participation and ongoing support for this study.

CONFLICTS OF INTEREST

Michael Abramson holds investigator initiated grants from Pfizer and Boehringer-Ingelheim for unrelated research. He has received assistance with conference attendance from and undertaken an unrelated consultancy for Sanofi. All other authors declare they have no conflicts of interest regarding this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13672

Comparison of regulatory B cells in asthma and allergic rhinitis

To the Editor,

B lymphocytes produce antibodies, act as antigen-presenting cells and produce cytokines.¹ They can be classified into functional subsets according to their distinct expression of surface markers and their profile of secreted cytokines. Regulatory B cells (Bregs), control excessive inflammatory responses primarily through secretion of IL-10. IL-10 inhibits proinflammatory cytokine production and supports regulatory T-cell differentiation.²⁻⁴ During the last decade, an essential role has been attributed to IL-10⁺ Bregs in limiting excessive immune reactivity in experimental models of infection, allergic inflammation, autoimmunity, tolerance, tumor growth, and organ transplantation.² An important in vivo functional role for human Bregs has been suggested by the finding that exacerbations of colitis and psoriasis take place after B-cell depletion therapy.⁵ An increase in peripheral CD19⁺CD25⁺CD71⁺CD73⁻ Bregs upon allergen-specific immunotherapy of patients has been linked to the suppression of IgE and upregulation of IgG4 production, as well as increased IL-10 production in allergen-specific T and B cells.³ So far, three different IL-10⁺ Breg subsets have been identified in humans. CD19⁺CD5⁺CD1d⁺ and CD19⁺CD24⁺CD38⁺ Bregs were shown to suppress Th1 cells,^{6,7} whereas CD19⁺CD25⁺CD71⁺CD73⁻ Bregs may play a role in allergen-specific immune tolerance. Moreover, Kamekura et al⁸ have demonstrated significant decrease of CD19⁺CD24^{hi}CD27⁺ B cells in allergic rhinitis and asthma. In contrast, another study reported an increase of CD19⁺CD24^{hi}CD27⁺ B cells and a decline of CD19⁺CD24^{hi}CD38^{hi} B cells in allergic rhinitis compared to healthy individuals.⁹ However, it is noteworthy that none of these studies assessed production of IL-10, which is a crucial feature of Bregs, as a marker of this population. Therefore, in view of this data and the lack of IL-10 measurement in the previous studies, we aimed to compare the percentages of IL-10-producing Breg subsets in peripheral blood from clinically well-characterized asthmatic, allergic rhinitis, and healthy 14-15 years old individuals (Table S1) using multicolor flow cytometry. Peripheral blood mononuclear cells were stained with antibodies specific for IL-10, CD1d, CD5, CD24, CD38, CD25, CD71, and CD73. Three types of Breg cells were studied according to the gating strategy shown in Figure 1A, based on the isotype controls (Figure S1). Since IL-10 has

been proposed as a key suppressive cytokine of Breg cells in vivo and in vitro,²⁻⁴ we aimed to compare the percentage of IL-10-producing B cells between patients with asthma, allergic rhinitis, and healthy controls at day 0. Donor-dependent variation in the total number of IL-10⁺ B cells (ranging between 1% and 24% of total B cells) was observed and there was no difference between the studied groups (Figure 1B). Further comparison of IL-10 expression within the three Breg populations revealed no difference in the percentage of CD19⁺IL-10⁺CD25⁺CD71⁺CD73⁻ (Figure 1C), CD19⁺IL-10⁺CD24⁺CD38⁺ (Figure 1D), and CD19⁺IL-10⁺CD5⁺CD1d⁺ (Figure 1E) Breg cells within or between patients with asthma and allergic rhinitis, and healthy controls at day 0. The comparison of total B cell percentage as well as naïve and memory B cells gated based on the expression of CD27 and IgD (Figure S2A) did not show any significant difference between the studied groups (Figure S2B). Despite the number of samples being limited, this observation may suggest that there is no deficiency in circulating Bregs in asthmatic and allergic rhinitis patients.

As we were interested in the response of B cells to B-cell-specific stimulants, we aimed to analyze if there is any difference in the capacity for the induction of IL-10 expression in the three groups after 3 days of stimulation with CpG oligonucleotides (CpG) or anti-B-cell receptor (α BCR) and CD40 ligand (CD40L). Cells were gated according to the strategy shown in Figure 2A. IL-10 production was significantly induced upon CpG stimulation in patients with asthma and healthy controls. There was a trend toward lower number of IL-10-producing Bregs in patients with allergic rhinitis. Therefore, it would require a larger patient cohort to determine whether patients with allergic rhinitis have a significant Breg deficiency. One can speculate that even the modest differences in Breg frequencies and function, may have an impact on systemic responses, as Bregs have been associated with the suppression of excessive inflammation, restoration of Th1/Th2 balance, inhibition of Th1 and Th17 differentiation, and induction of regulatory T cells, mediated not only through the release of soluble factors, but also via cell-to-cell contact.¹⁰ Moreover, a slight difference in circulating Breg frequencies could reflect a significant difference in tissue-infiltrating Breg cells. Further studies are needed to quantify Breg populations in respiratory mucosal tissues.

Online Repository material for:

Associations between cord serum per- and poly-fluoroalkyl substances (PFAS) and early life atopy and eczema: a nested case control study.

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Participant recruitment and ethical approval

Expectant mothers were approached during antenatal care visits at the former Mercy Hospital for Women (East Melbourne, Australia) and other private consulting suites. Children with a parent or older sibling with a history of allergic disease (asthma, eczema, allergic rhinitis or severe food allergy) were eligible for enrolment. Infants were born between 1990 and 1994. Parents provided informed consent, and the Mercy Hospital Human Ethics Committee granted ethical approval.

Analytical method:

Samples were analysed by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) using a Nexera HPLC (Shimadzu Corp., Kyoto, Japan) coupled to API5500 QTRAP mass spectrometer (Sciex, Melbourne, Australia) with electrospray ionization (ESI) interface operating in negative mode. Chromatographic separation of the analytes was achieved with a Gemini C18 column (50x2.0 mm, 4 µm; Phenomenex, Torrance, CA), maintained at 45 °C, with a flow rate of 0.3 mL/min and injection volume of 5 µL^[E1]. Mobile phases consisted of methanol:water (1:99, v/v) (A), and methanol:water (95:5, v/v) (B), with 5mM ammonium acetate in both phases. An isolator column (Phenomenex) was included inline directly after the mobile phase mixing chamber to delay the elution of solvent-derived background PFAS contamination. Data acquisition and processing was carried out using Analyst® TF 1.6 and MultiQuant™ software (Sciex). Target analytes were quantified via isotope dilution method and external calibration in the

absence of internal standards. Multiple transitions were measured for most analytes, but in some samples with low concentrations second transitions could not be confirmed as levels were close to the limit of quantification. However, results for these analytes have been included in this study as the single transitions in the samples were confirmed using native spikes and mass labelled standards. Where only a single transition was obtained for a subset of samples, the results are within the tables.

Correlations between specific PFAS were estimated using Pearson correlation coefficients. Correlations with $p < 0.05$ were considered significant. Visualisation of associations via a heatmap (Figure E1) was generated by calculating the p-value for the association between each PFAS, and each outcome. The statistical package “R” (version 3.5.1) was used to generate the heatmap.

Supplementary references

- ^{E1} Rotander A, Toms LM, Aylward L, et al. Elevated levels of PFOS and PFHxS in firefighters exposed to aqueous film forming foam (AFFF). *Environ Int.* 2015;82:28-34.

Table E1. Baseline characteristics of sample.

	Proportion (n/N)	Mean (sd) or median (IQR)
Female sex	50.0% (30/60)	
First born child	33.3% (20/60)	
Maternal ever eczema	45.0% (27/60)	
Paternal ever eczema	20.0% (12/60)	
Paternal smoking (ever)	31.7% (19/60)	
Paternal smoking (ever)	28.3% (17/60)	
Maternal completion of tertiary education	50.0% (30/60)	
Maternal age in years (sd)		30.7 (3.5)
Paternal age in years (sd)		33.6 (5.0)
Median weeks of breastfeeding (IQR)		36 (14-58)
Median duration of exclusive breastfeeding (IQR)		11.5 (1-18)

Table E2. Distribution of PFAS in cord serum samples and parameterisation of exposure.

PFAS	Accronym	Results above LOD	parameterisation	Mean (sd) - ng/mL	Median (IQR) ng/mL	Min – max ng/mL
Perfluorooctane sulfonic acid	PFOS	60	continuous	6.26 (2.74)	5.76 (4.25-7.66)	1.93-13.94
Perfluorooctanoic acid	PFOA	60	continuous	1.71 (0.79)	1.51 (1.07-2.39)	0.50-3.88
Perfluorohexane sulfonic acid	PFHxS	60	continuous	1.24 (1.37)	0.73 (0.52-1.07)	0.32-7.32
N-ethyl perfluoro-1-octanesulfonamidoacetic acid	NEtFOSAA**	60	continuous	1.31 (0.84)	1.09 (0.67-1.65)	0.37-4.38
Perfluoroundecanoic acid	PFOA**	59	continuous	0.33 (0.10)	0.30 (0.26-0.39)	0-0.72
Perfluorobutanesulfonic acid	PFBS	59	continuous	0.32 (0.12)	0.29 (0.24-0.35)	0-0.75
Perfluorononanoic acid	PFNA	57	continuous	0.28 (0.15)	0.25 (0.18-0.36)	0-0.92
Dichotomized (detected vs not detected)				Mean (sd)*	Median (IQR)*	Min – max*
N-methyl perfluoro-1-octanesulfonamidoacetic acid	NMeFOSAA **	40	dichotomous	0.77 (0.85)	0.40 (0.30-0.88)	0.19-3.80
Perfluoroheptanoic acid	PFHpA	23	dichotomous	0.34 (0.42)	0.23 (0.17-0.31)	0.12-2.20
Perfluorodecanoic acid	PFDA	21	dichotomous	0.15 (0.04)	0.15 (0.12-0.16)	0.09-0.30
N-ethyl perfluoro-1-octanesulfonamido)-ethanol	NEtFOSE	13	dichotomous	0.31 (0.12)	0.30 (0.23-0.42)	0.12-0.51
Perfluorohexanoic acid	PFHxA	9	dichotomous	0.41 (0.26)	0.33 (0.21-0.61)	0.12-0.82
Perfluorooctanesulfonamide	FOSA	7	dichotomous	0.07 (0.03)	0.06 (0.06-0.08)	0.03-0.13
PFAS excluded from analyses						
Perfluorobutanoic acid	PFBA	3	excluded			0.24-0.99
N-methyl perfluoro-1-octanesulfonamido-ethanol	NMeFOSE	2	excluded			0.19-1.55
Perfluorododecanoic acid	PFDoDA	2	excluded			0.08-0.12
Perfluoropentanoic acid	PFPeA	1	excluded			0.81
Perfluoro-n-tridecanoic acid	PFTriDA	0	excluded			-
Perfluorotetradecanoic acid	PFTeDA	0	excluded			-
Perfluorodecanesulphonate acid	PFDS	0	excluded			-
8:2 fluorotelomer sulfonate	8:2 FTS	0	excluded			
6:2 fluorotelomer sulfonate	6:2 FTS	0	excluded			
4:2 fluorotelomer sulfonate	4:2 FTS	0	excluded			

* Values based on those with a detectable concentration.

** Second transitions for a subset of samples could not be confirmed as levels were close to the limit of quantification. Second transition could not be quantified for 54/60 samples for PFOA, 14/60 for NEtFOSAA, and 27/60 for NMeFOSAA.

Table E3. Pearson correlation** matrix between assessed PFAS

	PFOA	PFNA	PFUnDA	PFBS	PFHxS	PFOS	NEtFOSAA
PFOA	1						
PFNA	0.46 (0.23,0.64)	1					
PFUnDA	0.03 (-0.23,0.28)	0.44 (0.20,0.63)	1				
PFBS	-0.16 (-0.40,0.10)	-0.01 (-0.27,0.25)	-0.07 (-0.32,0.19)	1			
PFHxS	0.23 (-0.02,0.46)	0.05 (-0.22,0.31)	0.08 (-0.18,0.33)	0.02 (-0.24,0.28)	1		
PFOS	0.53 (0.32,0.69)	0.45 (0.22,0.64)	0.29 (0.04,0.51)	-0.17 (-0.41,0.09)	0.38 (0.14,0.58)	1	
NEtFOSAA	0.13 (-0.13,0.37)	-0.05 (-0.31,0.21)	0.05 (-0.21,0.30)	-0.12 (-0.36,0.15)	0.06 (-0.20,0.31)	0.25 (-0.01,0.47)	1

* Correlations with $p < 0.05$ are in bold.

** Limited to those PFAS where detectable levels were identified in $\geq 95\%$ of samples

Figure E1. Heat map of adjusted associations between PFAS and eczema and atopy outcomes at 1 year of age, as per Table 2. Stronger associations are indicated by stronger red colours.

