

Exposure to a mixture of *per*- and polyfluoroalkyl substances modulates pulmonary expression of ACE2 and circulating hormones and cytokines

Zhao Yang^a, Katherine Roth^a, Jiahui Ding^b, Christopher D. Kassotis^{a,c}, Gil Mor^b, Michael C. Petriello^{a,c,*}

^a Institute of Environmental Health Sciences, Wayne State University, Detroit, MI 48202, USA

^b C.S Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, Wayne State University, Detroit, MI 48202, USA

^c Department of Pharmacology, School of Medicine, Wayne State University, Detroit, MI 48202, USA

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ABSTRACT

Genetic and environmental factors impact on the interindividual variability of susceptibility to communicable and non-communicable diseases. A class of ubiquitous chemicals, *Per*- and polyfluoroalkyl substances (PFAS) have been linked in epidemiological studies to immunosuppression and increased susceptibility to viral infections, but possible mechanisms are not well elucidated. To begin to gain insight into the role of PFAS in susceptibility to one such viral infection, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), male and female C57BL/6 J mice were exposed to control water or a mixture of 5 PFAS (PFOS, PFOA, PFNA, PFHxS, Genx) for 12 weeks and lungs were isolated for examination of expression of SARS-CoV-2-related receptors Angiotensin-Converting Enzyme 2 (ACE2) and others. Secondary analyses included circulating hormones and cytokines which have been shown to directly or indirectly impact on ACE2 expression and severity of viral infections. Changes in mRNA and protein expression were analyzed by RT-qPCR and western blotting and circulating hormones and cytokines were determined by ELISA and MESO QuickPlex. The PFAS mixture decreased Ace2 mRNA 2.5-fold in male mice ($p < 0.0001$), with no significant change observed in females. In addition, TMPRSS2, ANPEP, ENPEP and DPP4 (other genes implicated in COVID-19 infection) were modulated due to PFAS. Plasma testosterone, but not estrogen were strikingly decreased due to PFAS which corresponded to PFAS-mediated repression of 4 representative pulmonary AR target genes; hemoglobin, beta adult major chain (*Hbb-1*), Ferrochelatase (*Fech*), Collagen Type XIV Alpha 1 Chain (*Col14a1*), 5'-Aminolevulinic Synthase 2 (*Alas2*). Finally, PFAS modulated circulating pro and anti-inflammatory mediators including IFN- γ (downregulated 3.0-fold in females; $p = 0.0301$, 2.1-fold in males; $p = 0.0418$) and IL-6 (upregulated 5.6-fold in males; $p = 0.030$, no change in females). In conclusion, our data indicate long term exposure to a PFAS mixture impacts mechanisms related to expression of ACE2 in the lung. This work provides a mechanistic rationale for important future studies of PFAS exposure and subsequent viral infection.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) have been manufactured and widely used in various industries for their surfactant properties since the 1940's (Gluge et al., 2020; Prevedouros et al., 2006). PFAS are a family of synthetic chemicals made up of carbon-chain molecules that have been fluorinated (Buck et al., 2011), with over 4000 distinct varieties having been identified and around 250 currently being produced at globally relevant levels (Buck et al., 2021). These chemicals are very stable and highly resistant to degradation (Buck et al., 2011; Meegoda

et al., 2020) and are used in numerous industrial and consumer products, including non-stick cookware, food packaging, fabric, coatings and in aqueous film-forming foams used for fire extinguishing (Buck et al., 2011; Meegoda et al., 2020; Trier et al., 2011; Nickerson et al., 2020). For decades, long-chain "legacy" PFAS were the prevalent form being manufactured and utilized in products, including the well-studied perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). These legacy PFAS are made up of a hydrophobic head with a fluorinated hydrophilic tail, bearing a resemblance to fatty acids (Buck et al., 2011; Lindstrom et al., 2011). More recently, numerous studies exposing

* Corresponding author at: Wayne State University, 6135 Woodward Avenue, IBio 2128, Detroit, MI 48202, USA.

E-mail address: Michael.petriello@wayne.edu (M.C. Petriello).

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the various adverse health effects associated with PFOA and PFOS have led to the voluntary phase-out of these products 5–10 years ago in the United States. However, legacy PFAS were soon replaced with alternatives, such as tridecafluorohexane-1-sulfonic acid (PFHxS), as well as by the newest “emerging” PFAS, such as hexafluoropropylene oxide dimer acid (GenX) (Brase et al., 2021; Dodds et al., 2020; Wang et al., 2015). These “emerging” PFAS often exhibit shorter chains or fluoroether replacements, which are generally less bioaccumulative with increased excretion rates (Renner, 2006; Gannon et al., 2016). Studies have measured legacy, alternative, and emerging types of PFAS in water sources, soil, plants, animals, as well as humans around the globe (Domingo and Nadal, 2017; Domingo and Nadal, 2019; Calafat et al., 2007; Jha et al., 2021; Kato et al., 2011). Due to their high stability and resistance to degradation, PFAS are highly persistent in the environment (Domingo and Nadal, 2019; Zhang et al., 2022; De Silva et al., 2021; Brusseau et al., 2020). Humans, even among the general population, are widely exposed to PFAS through several exposure routes, the main ones being *via* drinking water and diet (Domingo and Nadal, 2019; Clara et al., 2008). Data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 reported that PFAS can be detected in the blood of 98% of adults in the U.S. Americans (Calafat et al., 2007).

Given the ubiquitous nature of PFAS that bioaccumulate in both the environment and in humans, numerous epidemiological studies have explored the adverse health effects associated with PFAS exposure (Grandjean and Clapp, 2015; Sunderland et al., 2019). Studies have linked PFAS exposure with effects ranging from immunotoxicity (DeWitt et al., 2012), dyslipidemia (Armstrong and Guo, 2019; Fenton et al., 2021; Blomberg et al., 2021), cardiometabolic diseases (Meneguzzi et al., 2021), to cancer (Steenland and Winquist, 2021) and developmental, reproductive, and endocrine disruptive effects (Rappazzo et al., 2017; Rickard et al., 2022). Previous studies have also identified the lung as one of the major target tissues for PFAS accumulation (Borg et al., 2010; Perez et al., 2013). Consequently, exposure to PFAS is associated with pulmonary diseases such as asthma in both human and animal studies. PFAS may exaggerate airway-related pathologies including Immunoglobulin E-related allergic responses (Fairley et al., 2007), decreased lung function in asthmatic children (Qin et al., 2017), and overall elevated pulmonary oxidative stress (Chen et al., 2012; Mao et al., 2013). Mechanisms of action may include systemic immunosuppression; PFAS exposures impact on post-vaccination antibody levels (Kielsen et al., 2016; Grandjean et al., 2017) and weaken antibody efficiency against multiple viruses including diphtheria, tetanus, and rubella (Bulka et al., 2021). In addition, PFAS exposures have recently been associated with increased susceptibility to multiple viral infections (Bulka et al., 2021).

Since PFAS has been shown to be immunosuppressive, a reasonable hypothesis is that PFAS possibly impacts on infection rate and/or severity of viral infections. Since 2020, the Coronavirus disease-2019 (COVID-19) pandemic caused by Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has induced severe global health burden and mortality. Recently, environmental factors including exposure to particulate matter (PM) 2.5, nitrogen dioxide, and ozone have been reported to be positively associated with hospitalizations and mortality of COVID-19 (SanJuan-Reyes et al., 2021; Weaver et al., 2022). Recent published epidemiological studies have also shown that PFAS exposures are positively correlated with susceptibility, severity and mortality of COVID-19 in Swedish, Chinese, Danish, and Italian populations (Grandjean et al., 2020; Nielsen and Joud, 2021; Ji et al., 2021; Catelan et al., 2021).

In addition to immunosuppression, PFAS exposures may impact on receptors and cofactors related to viral infection. Studies have identified the human plasma membrane protein Angiotensin-Converting Enzyme 2 (ACE2) as the target for internalization of SARS-CoV-2 virus (Ni et al., 2020). The severity of COVID-19 has been linked to multiple demographic variables including age, sex (male), and preexisting conditions such as diabetes and hypertension (Rashedi et al., 2020; Zheng

et al., 2020). ACE2 is the key enzyme in the renin-angiotensin system (RAS), in which ACE2 converts Angiotensin (Ang) II produced by ACE to Ang 1–7 (Burrell et al., 2004). The ratio between ACE2 and ACE is critical to balance between vasodilation and vasoconstriction and also impacts on inflammation *via* distinct receptors for each of the enzymatic products (Gaddam et al., 2014). Epidermiological studies have reported positive associations with PFAS and hypertension in adolescents and pregnant women, but it is unknown if PFAS-mediated modulation of ACE2 expression may be related to these observations (Averina et al., 2021; Erinc et al., 2021; Pitter et al., 2020; Huang et al., 2019). We therefore aim to determine whether pulmonary expression of ACE2 and related genes are altered when mice are exposed to 5 environmentally relevant PFAS (PFOA, PFOS, PFNA, PFHxS, and GenX) for 12 weeks. Furthermore, we investigated if PFAS-mediated impacts on ACE2 expression may be transcriptionally regulated through sex hormone receptors.

2. Materials and methods

2.1. Animal modeling and sample collection

The animal protocol was approved by the Institutional Animal Care and Use Committee at Wayne State University. The animal experimental design and protocols have been described previously in detail (Roth et al., 2021). In short, male and female wild-type C57Bl/6 J mice (Jackson Laboratories) were fed the Clinton/Cybulsky standardized diet; (Research Diets, New Brunswick, NJ, USA) and exposed to control vivarium water or water containing a mixture of 5 PFAS for 12 weeks: PFOS (Sigma-Aldrich, St. Louis, MO, USA, 98%), PFOA (Sigma-Aldrich, St. Louis, MO, USA, 95%), PFNA (Sigma-Aldrich, St. Louis, MO, USA, 97%), PFHxS (J&K Scientific, Beijing, China, 98%) and GenX (Synquest Laboratories, Alachua, FL, USA, 97%). Mice were assigned randomly to 4 experimental groups: vehicle & female ($n = 10$), PFAS & female ($n = 9$), vehicle & male ($n = 10$), and PFAS & male ($n = 10$). Each of the 5 PFAS chemicals was at a concentration of 2 mg/L in water. This exposure paradigm leads to circulating plasma levels of individual PFAS that mirror occupational exposures; For example, studies have found serum PFOA concentrations of 0.20 to 47.04 microgram/mL in workers (Costa et al., 2009). Our exposure paradigm leads to the following average plasma concentration (ug/mL) of the 5 PFAS in the female and male mice, respectively: PFOA (23 μ g/mL, 7 μ g/mL), PFOS (14 μ g/mL, 23 μ g/mL), PFHxS (22 μ g/mL, 26 μ g/mL), PFNA (34 μ g/mL, 41 μ g/mL), and GenX (0.2 μ g/mL, 0.1 μ g/mL) (Roth et al., 2021). Water consumption data (mL/mouse/day) has been reported previously (Roth et al., 2021); on average mice drank between 3 and 4.5 mL of water per day. All mice were fasted overnight and euthanized *via* carbon dioxide euthanasia at study conclusion. After perfusion by phosphate-buffered saline, all tissues were collected and immediately frozen and stored at -80°C prior to analysis.

2.2. Steroid hormones and angiotensin II quantification

Blood was collected at euthanasia and steroid hormones were extracted from 50 μ L of plasma using Ethyl Ether Anhydrous (Fisher Scientific) before being applied to commercial enzyme-linked immunosorbent assay (ELISA) kit. 17β -Estradiol and testosterone concentrations were determined by ELISA kits from Enzo Life Sciences (Cat# ADI-900-174 and ADI-900-065). Angiotensin II concentration was measured without extraction from plasma and was determined by ELISA kits from Enzo Life Sciences (Cat# ADI-900-204). The ELISA assays were conducted according to the manufacturer's instructions.

2.3. Plasma cytokine immunoassays

Plasma of the mice was used for analyzing the level of 9 inflammation-associated cytokines [Granulocyte-macrophage colony-

stimulating factor (GM-CSF), Interferon (IFN)- α , IFN- γ , Interleukin (IL)-10, IL-1B, IL-4, IL-6, Monocyte Chemoattractant Protein-1 (MCP-1), and Tumor necrosis factor (TNF α) in each group. A 96 well U-plex plate (U-PLEX Custom Biomarker Group 1 (ms) Assays) was used and cytokine levels were determined by the electrochemiluminescence platform (MESO QuickPlex SQ 120, MSD) following manufacturer's instruction. The raw data have been analyzed by the software provided by the manufacturer (Discovery Workbench 4.0, MSD) with default settings.

2.4. Immunoblotting

Lung tissues were homogenized in tris buffered saline. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. For Western blot analysis, the antibodies were diluted according to manufacturer's instructions. The antibodies were listed in the below table. The signal of the protein was captured by the ChemiDoc MP Imaging System (Biorad). Quantification of the protein relative level was conducted by ImageJ software (NIH, USA). *GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Gene name	Company	Catalog #
ACE2	Invitrogen	MA5-32307
ACE	Invitrogen	MA5-32741
GAPDH*	Cell Signaling	5174

2.5. Real-time polymerase chain reaction (RT-qPCR)

Total RNAs purified by Trizol reagent (Invitrogen) were reverse transcribed to cDNA for quantifying with an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System using SYBR Green (Applied Biosystems). Samples were analyzed as duplicates, and expression levels were calculated with the manufacturer's software using the $\Delta\Delta C_t$ method. The PCR primers are described in the below table. The house-keeping genes were listed at the bottom of the table marked in bold. Housekeeping genes are also denoted within figure legends.

Gene name	Forward (5' -> 3')	Reverse (5' -> 3')
ACE2	TGGTCTTCTGCCATCCGATT	CCATCCACCTCCACTTCTCTAA
ACE	AACATCCGTAACCATCACAGCCT	TCAAACACCATGTCCCCCATC
TMPRSS2	GAGAACCCTGTGTTCGTCTC	GCTCTGGTCTGGTATCCCTTG
ANPEP	CACCCCAACCTTCGGTCTAC	GTTTGTCCGCTTCGTTCCACC
ENPEP	CACAAAGTTACTCCGGGCTC	TACCGGAATACTTGCCTCC
DPP4	ACAGTCATGAGCAGAGCTGA	CCGTGTACCACATTCGCTGG
FOXA2	GAGGGCTACTCTCCGTGAG	TGACATGTTTCATGGAGCCCG
HNF1B	AGCCAGTCGGTTTTACAGCA	CCCAGACTGTGATCTGCATT
SIRT1	TGACCGATGGACTCCTCACT	ATTGTTCCGAGGATCGGTGCG
ESR1	CTGGCTACGTCAAGTCGGTT	AGGTGCTGGACAGAAACGTG
ESR2	TCTTTGTCTCCAGACCTCGTTC	TGGGACAGCACTCTCTGCTCT
AR	AAAGAGCCGCTGAAGGGAAA	GGAGACGACAAGATGGGGCAA
PGR	TATGGCGTGCCTTACCTGTGG	TGCCAGCTGACAACACTTT
NQO1	TCTCTGGCCGATTACAGAGTG	CCAGACGGTTTCCAGACGTT
VEGFA	TGCTGTAACGATGAAGCCCT	TGAACAAGGCTCAGATGATTTT
C3	CCCAATGTCTACTCGGCTGG	TCCTTACTGGCTGGAATCTTGA
FOXA1	AACAGCTACTACGCGGACAC	GCTCGTGGTCAATGGTGTTC
HBB-B1	GCACCTGACTGATGCTGAGAA	TTCATCGGCGTTCACCTTTCC
FECH	AGCTCAACCAGAAAGGAGAAAG	CTTCTCCAAGGGTTTCGGGG
COL14A1	CAGGCTTGTGCGCAATTCT	TGTACTGTGCCAAGCCGATT
ALAS2	ACCACCCGAAGCCATTCATT	GGTAGGCATTAGCCCAACGA
ACTB	GCCACTGTGAGTCCGCT	GATACCTCTCTGCTCTGGGC
GAPDH	CAAGGAGTAAGAAACCTGGACC	CGAGTTGGGATAGGGCCCTCT

2.6. Statistical analysis

The sample size (n) of biological independent samples is indicated in the related result part. The data were analyzed by the two way Analysis of variance (ANOVA), followed by the Sidak method post-hoc test for multiple comparisons. Significant *p* values are indicated by asterisks or crosses in the figures. *p* < 0.05 (*), *p* < 0.01 (**) in the Female groups

and *p* < 0.05 (+), *p* < 0.01 (‡) in the Male groups were considered statistically significant. Statistical analyzes were performed with GraphPad Prism 6 software. Exploratory multivariate correlation analyses were performed using JMP® Pro, Version 14.0. (SAS Institute Inc., Cary, NC) with spearman correlations and *p*-values reported.

3. Results

3.1. Exposure to a PFAS mixture modulates ACE2/ACE ratio and other SARS-CoV-2-related genes in lung

To begin to gain insight into pulmonary impacts on expression of ACE2 and ACE due to ingestion of a PFAS mixture, lung tissue from male or female C57BL/6 J mice exposed to vehicle or PFAS through the drinking water was analyzed by RT-qPCR to measure mRNA expression changes of target genes. Sexual dimorphism was observed in the expression of *Ace2*. PFAS mixture decreased *Ace2* 2.5-fold in male mice (*p* < 0.0001), but no change in females was observed (*p* = 0.8676) (Fig. 1A). *Ace* expression was not changed in either sex due to PFAS, thus the ratio of *Ace2/Ace* expression was significantly increased by PFAS only in males (2.8 fold, *p* = 0.0022) (Fig. 1A).

Next, we confirmed these changes of ACE2/ACE ratio in males at the protein level by western blotting (*n* = 5 per group). In the PFAS treated male mice, ACE2/ACE ratio showed 1.57 fold decrease (*p* = 0.0210) compared with vehicle group (Fig. 1B). Finally, to determine if these changes in ACE2/ACE at the lung may be mirrored by parallel changes in plasma levels of Angiotensin II, a major enzymatic product of ACE, we observed an unexpected decrease in AngII irrespective of sex (Fig. 1A).

Although we focus primarily on impacts of ACE2/ACE here, we also examined gene expression changes due to ingestion of PFAS mixture on other genes implicated in COVID-19 infection (Fig. 1C). Serine protease transmembrane protease, serine 2 (TMPS2) is a crucial mediator for cell entry of SARS-CoV-2 through SARS-spike protein binding to ACE2 (Hoffmann et al., 2020). alanyl aminopeptidase (ANPEP), Glutamyl aminopeptidase (ENPEP) and Dipeptidyl peptidase-4 (DPP4) have been considered as the coreceptors of ACE2 for SARS-CoV-2 entry (Qi et al., 2020; Lange et al., 2020). mRNA levels of *Anpep* and *Enpep* were down-regulated by PFAS treatment in both sexes. *Anpep* expression was reduced 1.55 and 1.26 fold in the males (*p* < 0.0001) and females (*p* = 0.0448), respectively. *Enpep* expression was reduced 1.69 and 1.44 fold in the males (*p* < 0.0001) and females (*p* = 0.0009), respectively. A similar decreasing trend was observed in PFAS exposed male mice for *Tmprss2* (*p* = 0.3155) and *Dpp4* (*p* = 0.1764).

We hypothesized that PFAS may impact on ACE2/ACE expression through modulation of transcription factors previously shown to impact on transcription of *Ace2*, including hepatocyte nuclear factor 1 (HNF1) (Senkel et al., 2005), Sirtuin 1 (SIRT1) (Clarke et al., 2014) and Forkhead Box A (FOXA)2 (Pedersen et al., 2017). However, our RT-qPCR results suggested that none of these gene showed significant sex specific changes due to PFAS treatment (Fig. 1D). Overall, expression of *Foxa2* increased with PFAS (*p* = 0.0396), but similar effects were absent in the other two transcription factors.

3.2. Endocrine disruption activity of PFAS may impact on ACE2 expression

With the observation that PFAS induced down-regulation of pulmonary ACE2 primarily in male mice, and that transcription of *ACE2* has been reported to be regulated by sex hormones (Stelzig et al., 2020; Baratchian et al., 2021), we next investigated involvement of sex hormones in PFAS mediated expression changes of ACE2. ELISA results of plasma 17 β -estradiol (E2) and testosterone (T) levels indicated that T but not E levels were strikingly decreased in the PFAS exposed mice (Fig. 2A). Overall, irrespective of sex, PFAS treatment significantly decreased plasma T level (*p* = 0.0265) with males exhibiting a 35.7 fold decrease and females exhibiting a 62.8 fold decrease. We further

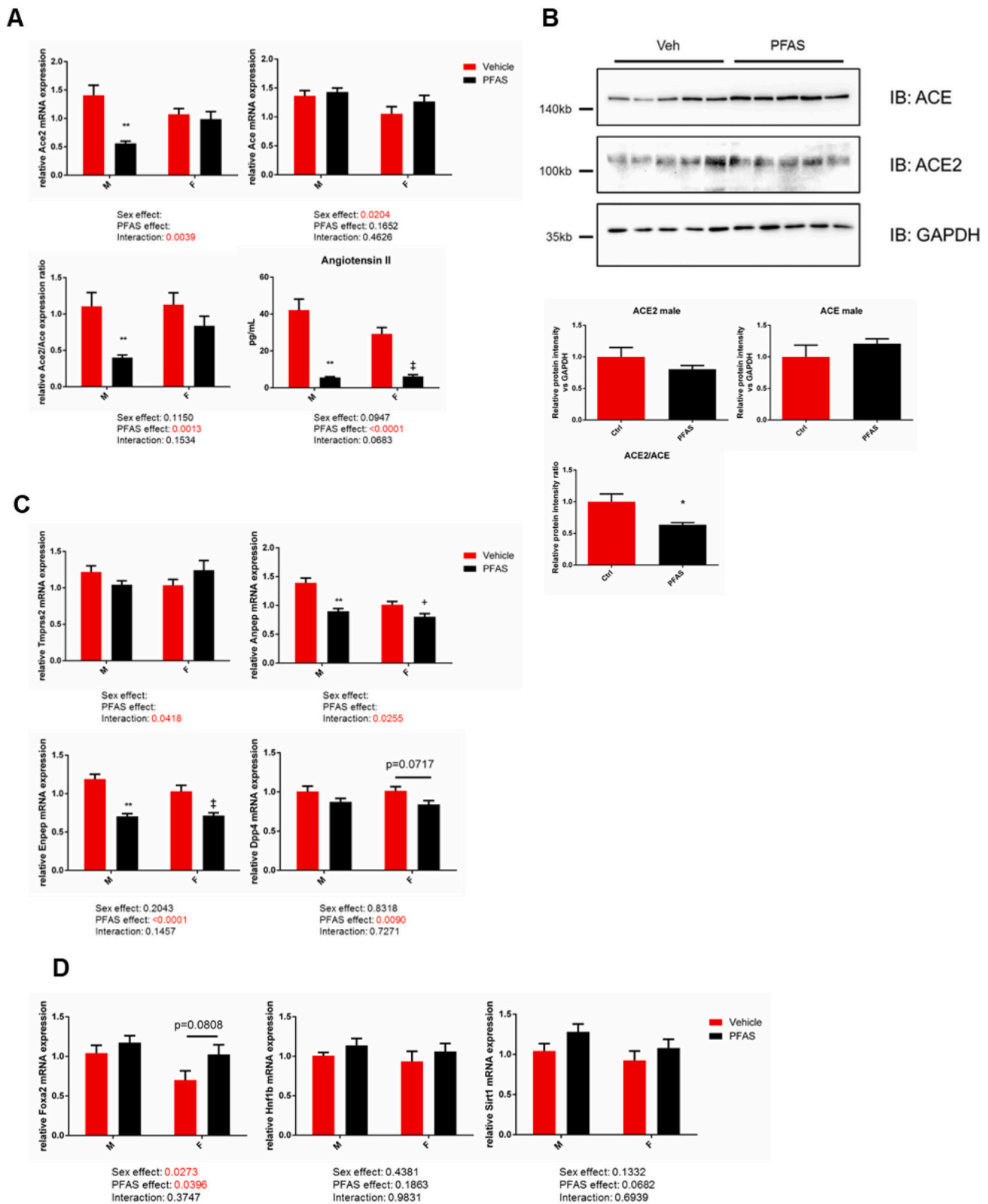


Fig. 1. PFAS exposure modulates pulmonary expression of COVID-19 associated receptors. Male (M) and female (F) C57BL/6 J mice were exposed to vehicle water (Vehicle) or the PFAS mixture (PFAS) for 12 weeks. Sample size for all results has been indicated here unless otherwise mentioned: F/vehicle ($n = 10$), F/PFAS ($n = 9$), M/vehicle ($n = 10$), and M/PFAS ($n = 10$). A. Pulmonary mRNA expression of *Ace2* (upper left) and *Ace* (upper right) and their ratio (lower left) are shown. Plasma Angiotensin II level has been shown on the lower right panel. B. Protein levels of ACE, ACE2 and housekeeping gene GAPDH in the male mice lungs are indicated ($n = 5$). Lower panels indicates quantification of immunoblotting results. C. Pulmonary mRNA expression of *Tmprss2*, *Anpep*, *Enpep* and *Dpp4* are shown. D. Pulmonary mRNA expression of *Foxa2*, *Hnf1b* and *Sirt1* are shown, normalized by GAPDH level. For all panels showed in this article, data are presented as means \pm SEM. When compared with male controls, * $p \leq 0.05$; ** $p \leq 0.01$. When compared with female controls, † $p \leq 0.05$; ‡ $p \leq 0.01$.

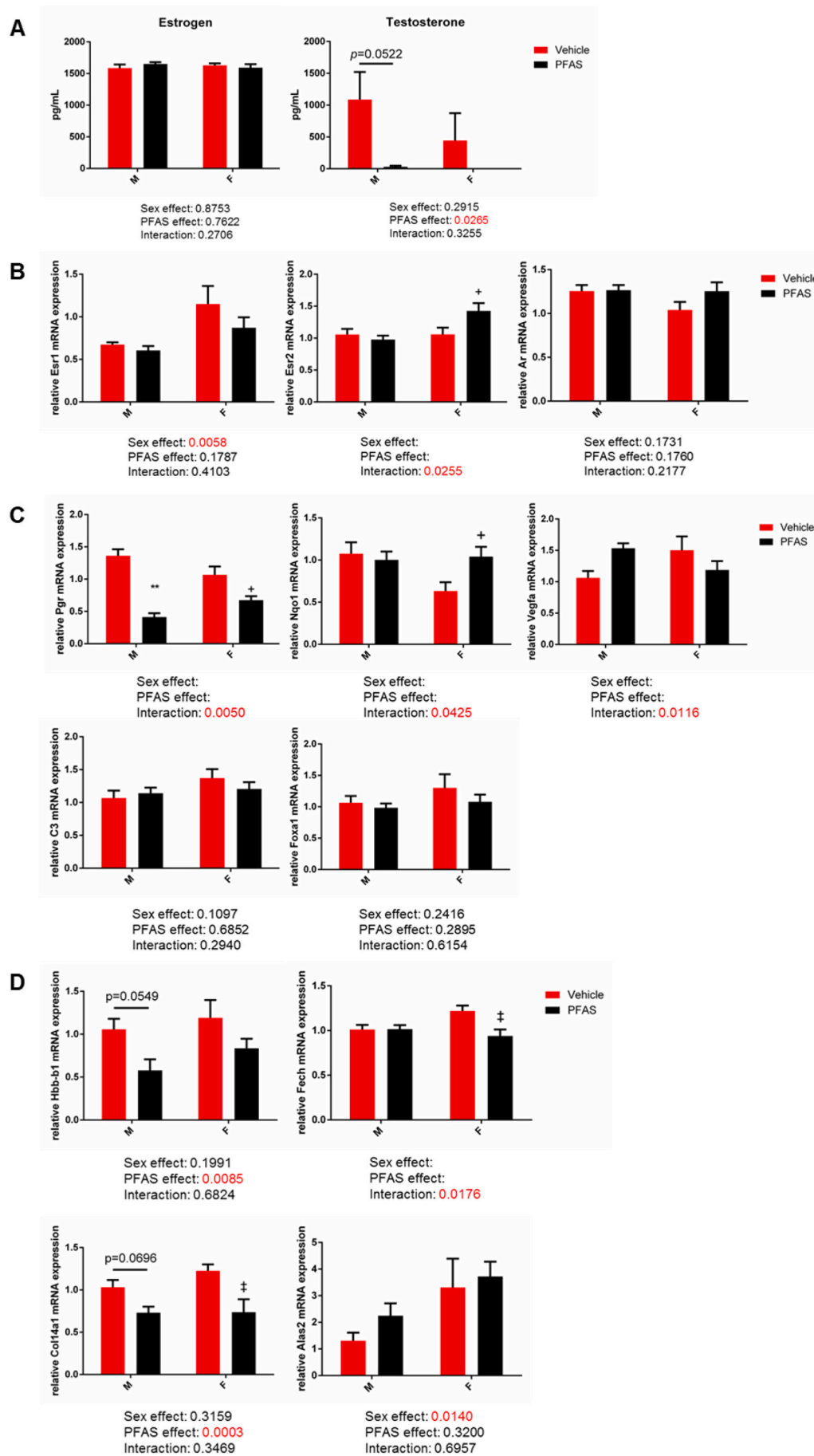


Fig. 2. PFAS decreases androgen signaling, which is a regulator of ACE2 expression. A. Plasma level of Estrogen has been shown on the left panel. Plasma level of Testosterone has been shown on the right panel (n = 5). B. Pulmonary mRNA expression of *Esr1*, *Esr2* and *Ar* are shown. C. Pulmonary mRNA expression of *Pgr*, *Nqo1*, *Vegfa*, *C3* and *Foxa1* are shown, normalized by GAPDH level. D. Pulmonary mRNA expression of *Hbb-b1* and *Fech* are shown. For all panels showed in this article, data are presented as means ± SEM. When compared with male controls, * $p \leq 0.05$; ** $p \leq 0.01$. When compared with female controls, † $p \leq 0.05$; ‡ $p \leq 0.01$.

analyzed sex specific mRNA level of estrogen receptors (*Esr1* and *Esr2*) and androgen receptor (*Ar*) with or without PFAS treatment. The result shows that other than *Esr2* level in the female mice, which has shown 1.35 fold increase in PFAS group ($p = 0.0227$), effects of PFAS treatment on expression of sex hormone receptors were insignificant (Fig. 2B).

In addition to levels of the hormone receptors themselves, we next examined impacts of PFAS exposure on the activity of these receptors by examining mRNA expression of target genes. We determined that ESR target gene expressions were modulated by PFAS mixture exposure (Fig. 2C). Expression of progesterone receptor (*Pgr*) was down regulated in PFAS treated group in both sexes (male 3.30 fold, $p < 0.0001$; female 1.58 fold, $p = 0.0108$). NAD(P)H Quinone Dehydrogenase 1 (*Nqo1*) was increased in the female PFAS exposure mice (1.64 fold, $p = 0.0351$). Three ESR target genes, Vascular Endothelial Growth Factor A (*Vegfa*), Complement C3 (*C3*) and *Foxa1*, showed no significant change in the PFAS exposure group.

Focusing next on our observation of decreased circulating testosterone due to PFAS, we examined expression changes for 4 representative pulmonary AR target genes: hemoglobin, beta adult major chain (*Hbb-b1*), Ferrochelatase (*Fech*), Collagen Type XIV Alpha 1 Chain (*Col14a1*), 5'-Aminolevulinic Synthase 2 (*Alas2*). Among the four genes, effect of PFAS exposure on the expression of *Fech* was dependent on sex (interaction $p = 0.0176$); expression of *Hbb-b1* (PFAS effect $p = 0.0085$) and *Col14a1* (PFAS effect $p = 0.0003$) were significantly affected by exposure but not by sex; expression of *Alas2* was associated with sex only (sex effect $p = 0.0140$) (Fig. 2D). Interestingly, Sidak post-hoc test showed PFAS treatment induced 1.83 fold decrease on *Hbb-b1* in male mice ($p = 0.0549$), 1.30 fold decrease on *Fech* in female mice ($p = 0.0031$), 1.41 fold and 1.66 fold decrease on *Col14a1* in male ($p = 0.0696$) and female ($p = 0.0029$) mice, respectively.

3.3. PFAS impacts on circulating cytokine levels in a sex specific manner

Finally, to analyze whether PFAS-driven sex-differentiated down-regulation of ACE2 is associated with inflammatory factors, we analyzed plasma level of 9 inflammation-associated cytokines, including GM-CSF, IFN- α , IFN- γ , IL-10, IL-1B, IL-4, IL-6, MCP-1, and TNF α , by using commercial multiplex assays. Plasma levels of IFN- γ were downregulated 3.0-fold in PFAS-exposed females compared to vehicle ($p = 0.0301$) and 2.1-fold in PFAS-exposed males compared to vehicle ($p = 0.0418$); p value of overall exposure effect on IFN- γ was 0.0021 (Supp Fig. 1A). Plasma levels of IL-10 were not impacted by exposure in either sex (Supp Fig. 1B). Plasma levels of IL-6 were upregulated 5.6-fold in PFAS-exposed males compared to vehicle ($p = 0.0301$) and no significant changes were observed in females; p value of overall exposure effect on IL-6 was 0.0078 and p value of interaction was 0.0221 (Supp Fig. 1C). In contrast to IL-6, plasma levels of MCP-1 were upregulated 2.2-fold only in PFAS-exposed females compared to vehicle ($p = 0.0386$); p value of overall exposure effect on MCP-1 was 0.0075 (Supp Fig. 1D). The other 3 cytokines, IFN- α , IL-1B and TNF α , did not show significant changes either by sex or treatment (Supp Fig. 1E-G). Results for GM-CSF and IL-4 are not reported because concentrations of these 2 cytokines were below the standard curve.

4. Discussion

Here, we show that ingestion of PFAS leads to down-regulation of pulmonary ACE2 expression which coincides with perturbation of androgen receptor signaling and cytokine profiles. Interestingly, there were observed strong sexual dimorphic effects, with PFAS impacting on our endpoints of interest more strongly in male mice. We investigated sex hormone signaling and identified testosterone-androgen receptor pathway has been down-regulated by PFAS exposure. Within this discussion, we will discuss 1) the roles of ACE2 in the RAS, inflammation and development of COVID-19, 2) sexual dimorphic expression of ACE2 and its association with sex hormone signaling, and 3) association

between PFAS exposure and sex hormone signaling.

ACE2 has been considered as a critical regulating enzyme in RAS, in which the major role in the RAS is to convert angiotensin (Ang) II into Ang 1–7 (Burrell et al., 2004; Patel et al., 2016). In contrast with ACE mediated vasoconstriction and proinflammation effects, the signaling of ACE2 activates vasodilatory and anti-inflammatory pathways through an endogenous orphan receptor, Mas and its related receptors, such as MAS Related GPR Family Member D (MrgD) (Patel et al., 2016; Guignabert et al., 2018). Aside from the classical effect of blood pressure regulation RAS components, especially ACE and ACE2, have been long considered as regulators in the pathogenesis of lung injury (Hamming et al., 2007). For example, global loss of ACE2 aggravates acid aspiration or sepsis-induced acute lung injury pathology in mice (Imai et al., 2005). Another example is in mice with ACE2 specific knockout in lung epithelial cells, which displayed increased neutrophil infiltration and lung injury when undergoing a model of bacterial pneumonia (Sodhi et al., 2019). An ACE2 enhancer, diminazene aceturate has been reported to protect against pulmonary hypertension (Shenoy et al., 2013) and allergic airway inflammation (Dhawale et al., 2016) in animal models. As a result, ACE2 has been targeted for prevention of pulmonary hypertension and acute respiratory distress syndrome in clinical trials (Hemnes et al., 2018; Khan et al., 2017). Furthermore, downregulation of ACE2 expression in the alveolar type II cells can impair lung elasticity and eventually lead to fibrosis (Verdecchia et al., n.d.). Since ACE2 is an AngII scavenger enzyme, increased AngII is expected to be observed when ACE2 is reduced. An unexpected observation in our work is that serum level of the ACE2 substrate, AngII was significantly reduced by PFAS treatment (Fig. 1A). One possibility is that expression of ACE2 in other tissues are oppositely regulated as in the lung by PFAS treatment. Other tissues to investigate in future studies include gastrointestinal tract, bladder and kidney, which all have been shown to express ACE2 in both human (Li et al., 2020; Atlas, 2022) and mice (Gemhardt et al., 2005). In addition to the well established roles of ACE2 in hypertension, binding of SARS-Cov-2 spike protein to ACE2 has recently been shown to initiate viral uptake, facilitated by various plasma membrane proteins as “coreceptors”. Some of the previously reported coreceptors of ACE2 for SARS-CoV-2 entry (TMPRSS2, ANPEP, ENPEP and DPP4) (Qi et al., 2020; Lange et al., 2020) were modulated by PFAS exposure; Anpep and Enpep displayed PFAS induced down-regulation regardless of sex (Fig. 1C).

Previous evidence has suggested that circulating cytokines may be related to severity of respiratory illnesses (Branchett and Lloyd, 2019) and may impact on ACE2 expression (Rodrigues Prestes et al., 2017). Also, PFAS have been shown to be associated with cytokine changes in epidemiological studies (Nian et al., 2022; Salihovic et al., 2020). We therefore analyzed plasma levels of cytokines in both sexes after PFAS exposure. Our data suggested that PFAS exposure induced down-regulation of IFN- γ in both sexes, but upregulation of IL-6 in male and MCP-1 in female, respectively (Supp Fig. 1). Consistent with our data, upregulation of cytokines including IL-6, MCP-1, and TNF α have been observed in ACE2 knockout mice (Thomas et al., 2010), suggesting possible regulatory effect of ACE2 on cytokine levels. Furthermore, pathological factors such as SARS-CoV-2 induced ACE2 depletion promotes pro-inflammatory reactions called “cytokine storm” (Banu et al., 2020). IFN- γ has played critical roles in mitigating SARS-related cytokine storm (Chua et al., 2020; Borden et al., 2007; Galbraith et al., 2022; Gadotti et al., 2020) and PFAS have been linked to lower IFN levels in humans (Abraham et al., 2020). Therefore, our data suggest the possibility that exposure to PFAS may predispose an individual to severe outcomes due to downregulation of protective IFNs.

Our results show sexual dimorphic change of ACE2 expression in the PFAS exposed mouse lung, and our further studies have suggested PFAS-regulated androgen signaling should be considered as one of the major factors which contributes to sexual dimorphic change in ACE2 (summarized in Fig. 3). Interestingly, previous evidence has indicated a sex-biased expression of ACE2. ACE2 is a typical gene which escapes X-

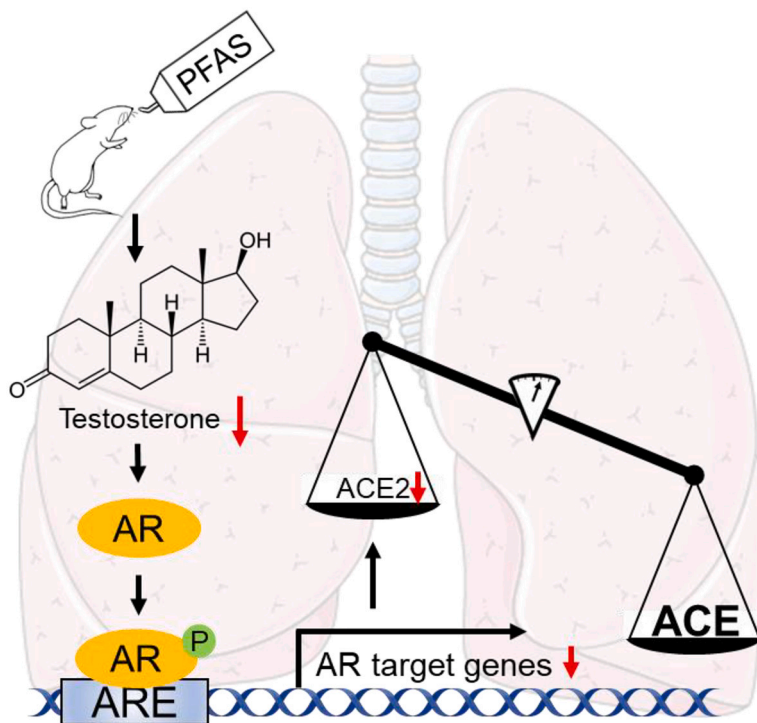


Fig. 3. Overview of possible mechanism linking PFAS exposure to pulmonary downregulation of ACE2 through sex hormone signaling. PFAS exposure decreases circulating testosterone levels, and parallel downregulation of the pulmonary target genes of androgen receptor including *Ace2*. Perturbation of the ACE2/ACE balance may lead to pathological outcomes related to inflammation and circulatory diseases. Parts of the figure were generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

chromosome inactivation in females (Carrel and Willard, 2005) which could lead to an expected higher expression of this gene in females. However, although heterogeneous pattern of male/female expression is observed, most tissues actually show higher expression of ACE2 in men (Tukiainen et al., 2017). Sexually dimorphic expression of ACE2 becomes an important topic given the ongoing COVID-19 pandemic, since worse outcomes and increased lethality have been observed in male patients (Jin et al., 2020; Penna et al., 2020). However, inconsistent conclusions have been drawn in various studies whether pulmonary expression of ACE2 increases or remains unchanged in male compared with female cohorts. Two *in silico* studies (including a preprint study) found no significant difference in human male/female lung cells (Asselta et al., 2020; Cai, 2020). Another study, however, analyzed sex dependent expression of ACE2 in human type II pneumocyte *in silico*, and they found expression was increased in males (Song et al., 2020). Moreover, by analyzing RNA-seq datasets, Li et al. indicated in their study that sexual dimorphic expression of ACE2 was tissue specific, which is associated with levels of immune signatures (Li et al., 2020). Liu et al. (Liu et al., 2010) described a similar effect that sex differences in ACE2 activity were observed in mouse kidney, but not in the lung and heart. As ACE2 and its enzymatic products are critical mediators of hypertension, it is interesting that several epidemiological studies have suggested positive associations between serum PFAS concentrations and onset of hypertension (Pitter et al., 2020; Khalil et al., 2018; Bao et al., 2017; Ma et al., 2019; Min et al., 2012; Yang et al., 2018; Liao et al., 2020). Interestingly, sex-specific effects have been observed in multiple studies, including two studies analyzing younger participants which show stronger associations in males (Pitter et al., 2020; Ma et al., 2019). Sexually dimorphic expression of ACE2 is largely regulated by sex hormones and their receptors. Expression and activity of ACE2 have been reported to be down-regulated by estrogen receptor (ESR) signal in multiple cell types (Stelzig et al., 2020; Liu et al., 2010; Bukowska et al., 2017; Kalidhindi et al., 2020). However, effect of estrogens on expression of ACE2 are somewhat controversial, and are tissue, species, and model dependent.

Testosterone/androgen receptor (AR) on the contrary, have not been suggested to induce expression of ACE2 until recent findings. Serum

testosterone was reduced in both SARS-CoV-1 infected mice (Channappanavar and Perlman, 2017) and in COVID19 patients (Corona et al., 2020), in whom expression of ACE2 has been reported as well. Later studies discovered mRNA expression of ACE2 in the lung was associated with AR activity by using a castrated male mouse model (Qiao et al., 2020; Chen et al., 2020). The conclusion has been confirmed by Baratchian et al. that protein levels of ACE2 were associated with human pulmonary expression of AR, and an AR antagonist suppressed ACE2 expression in human patients (Baratchian et al., 2021). Taken together with our data, AR can positively regulate mRNA expression of ACE2, at least in the mouse lung cells. Our data also showed AR target genes *Hbb-1*, *Fech* and *Col14a1* were decreased in the PFAS treated group (Fig. 2D). Therefore, further studies are required to understand which other mechanisms offset PFAS induced and AR mediated downregulation of ACE2 in the female mouse lung, and whether this effect of PFAS is mirrored in humans. Previous studies have indicated associations between PFAS and sex hormones. *In vitro* experiments have suggested that certain PFAS such as PFOA and PFOS can modulate ER activity in human cell lines (Behr et al., 2018). In some fish species, exposure to PFAS increases ESR2a expression (Rodriguez-Jorquera et al., 2019). Likewise, PFAS can significantly increase dihydrotestosterone-activated AR activity in a dose dependent manner (Behr et al., 2018). Interestingly, impairment effects of PFAS on sex hormones/receptors have been reported elsewhere. PFOS exposure has been negatively associated with E2 levels in human and mouse models (Feng et al., 2015; Barrett et al., 2015). However, exposure to PFAS mixtures has been associated with decreased serum estradiol concentrations in young men (Luo et al., 2021), and with increased serum estradiol concentrations in adolescents (Zhou et al., 2017), supporting a potential role of PFAS on ER signaling. In our model of occupational PFAS exposure, we did not observe impacts on ER signaling in the lung or with circulating estrogen levels. Effects on androgen receptor signaling have also been reported following exposure to PFAS. Previous *in vitro* research showed that PFOS and other PFAS positively or negatively modulated T-activated AR translocation and transactivation (Behr et al., 2018; Tachachartvanich et al., 2022; McComb et al., 2020). In females, most publications report no associations between PFAS and T,

with one exception from adolescent participants where PFAS levels were inversely associated with serum T (Ding et al., 2020). On the contrary, preclinical and epidemiological observations have indicated that exposure to PFAS lowers T level and impairs reproductive health in males (Tarapore and Ouyang, 2021; Di Nisio et al., 2019). In our study, we observed multiple endpoints that displayed PFAS-mediated effects especially or only in male mice, which is consistent with the literature on PFAS modulating AR signaling. A recently published study evaluated *in vitro* transactivation effect of 142 PFAS on 81 transcription factors (Houck et al., 2021), and reported impacts on multiple known and novel transcription factor reacted to PFAS, including ER. Although their data showed PFAS treatment induced no transcriptional activity on AR, it's possible that PFAS inhibits rather than promotes AR signaling as we showed in Fig. 2D; alternatively, it could be an *in vitro*-*in vivo* difference. Aside from transcriptional regulation, epigenetic regulation and mRNA decay mechanism may be involved in PFAS induced expression change of genes of target. Ahmad et al. have reported recently that short term, high dose (20 mg/kg/day) exposure of PFOA leads to about 1.6 fold up-regulation of pulmonary mRNA level of *Ace2* in CD1 mice (Ahmad et al., 2021), which is hypothesized to be mediated by DNA methylation, since expression of the major DNA methylation regulators have been down-regulated by PFOA treatment. This result is opposite to our main findings and may be related to major differences in exposure duration, single versus mixture exposures, or genetic background of mice.

Several limitations should be considered in this current study. This work used tissues from a previously published study focused on PFAS-mediated effects on lipid metabolism and liver injury (Roth et al., 2021). Therefore, impacts on the lung were not a primary endpoint and thus lung tissues were not preserved for histological analyses. Additionally, the original study was completed during the COVID-19 pandemic lockdown and longitudinal blood collection, useful for the trajectory of hormones and cytokines, was not possible. Additionally, although mixture studies may be more environmentally relevant, only one dose of PFAS was utilized herein and parallel studies using individual PFAS were not completed. Finally, studies that include castrated male mice with/without hormone replacement and studies that utilize humanized ACE2 mice will be important future endeavors for studying the impact of PFAS exposures on susceptibility to viral infections.

In conclusion, our results indicate long term ingestion of an environmentally relevant PFAS mixture leads to imbalance of pulmonary ACE2/ACE ratio as well as modulation of other Sars-Cov-2-related genes. We have presented the possible mechanism that PFAS mediates these changes through disruption of androgen receptor signaling. Future studies are needed to understand the detailed mechanism of this endocrine disrupting ability of PFAS and if exposure to PFAS increases the risk or severity of COVID-19 and other related respiratory illnesses. Furthermore, epidemiological studies that measure circulating PFAS, hormone levels, ACE2 activity, and measures of virulence would be beneficial to provide additional support to our primary hypotheses.

Author contributions

Zhao Yang: Conceptualization, Methodology, Writing - original draft. Katherine Roth: Methodology, Writing - review and editing. Jiahui Ding: Writing - review and editing. Christopher Kassarotis: Writing - original draft, Writing -Review and Editing. Gil Mor: Writing - review and editing. Michael Petriello: Writing- Review and editing, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2022.116284>.

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