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Research article

PFAS and their association with the increased risk of cardiovascular disease in postmenopausal women

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Abstract

Cardiovascular diseases (CVDs) are one of the major causes of death globally. In addition to traditional risk factors such as unhealthy lifestyles (smoking, obesity, sedentary) and genetics, common environmental exposures, including persistent environmental contaminants, may also influence CVD risk. Per- and polyfluoroalkyl substances (PFASs) are a class of highly fluorinated chemicals used in household consumer and industrial products known to persist in our environment for years, causing health concerns that are now linked to endocrine disruptions and related outcomes in women, including interference of the cardiovascular and reproductive systems. In postmenopausal women, higher levels of PFAS are observed than in premenopausal women due to the cessation of menstruation, which is crucial for PFAS excretion. Because of these findings, we explored the association between perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), and perfluorobutanesulfonic acid in postmenopausal women from our previously established CVD study. We used liquid chromatography with tandem mass spectrometry, supported by machine learning approaches, and the detection and quantification of serum metabolites and proteins. Here, we show that PFOS can be a good predictor of coronary artery disease, whereas PFOA can be an intermediate predictor of coronary microvascular disease. We also found that the PFAS levels in our study are significantly associated with inflammation-related proteins. Our findings may provide new insight into the potential mechanisms underlying the PFAS-induced risk of CVDs in this population.

This study shows that exposure to PFOA and PFOS is associated with an increased risk of cardiovascular disease in postmenopausal women. PFOS and PFOA levels correlate with amino acids and proteins related to inflammation. These circulating biomarkers contribute to the etiology of CVD and potentially implicate a mechanistic relationship between PFAS exposure and increased risk of cardiovascular events in this population.

Key words: PFAS; cardiovascular disease; postmenopausal women; human serum.

Cardiovascular disease (CVD) is one of the leading causes of death globally (Roth et al. 2020; Kesar et al. 2022; Hacker 2024). In addition to traditional risk factors such as unhealthy lifestyles (smoking, obesity, sedentary) and genetics, exposure to persistent environmental contaminants may also influence CVD risk (Bhatnagar 2006, 2017; O'Toole et al. 2008). Per- and polyfluoroalkyl substances (PFASs) are a class of highly fluorinated chemicals used in household consumer products (Trudel et al. 2008; Lindstrom et al. 2011; Bečanová et al. 2016; Hill et al. 2017; Schaider et al. 2017), which persist in our environment for years (Buck et al. 2011; Xu et al. 2020a; Sims et al. 2022). PFAS has been detected in the blood of more than 90% of the US population (Calafat et al. 2007, 2019), raising many health concerns that are now linked to endocrine disruptions and related outcomes in women (Zhou et al. 2017; Ding et al. 2020b), including the prevalence of CVDs (Shankar et al. 2012; Huang et al. 2018; Xu et al. 2020b).

Higher PFAS concentrations are found in men compared with menstruating woman (Calafat et al. 2007; Fromme et al. 2007; Bartolomé et al. 2017). This is thought to be due to gender differences in PFAS routes of elimination such as pregnancy or parity (Kato et al. 2014; Oh et al. 2022; Wise et al. 2022), breastfeeding (Kato et al. 2014; Bartolomé et al. 2017; Kim et al. 2020; Oh et al. 2022), and menstruation (Ding et al. 2020a; Upson et al. 2022; Wise et al. 2022). Studies did not report these differences in postmenopausal women, and men. Women of older age groups have similar PFAS concentrations (Harada et al. 2005; Salihovic et al. 2015; Bartolomé et al. 2017). Postmenopausal women were reported to have higher levels of PFAS than premenopausal women due to the cessation of menstruation, which is an important way of PFAS excretion (Wong et al. 2014). The postmenopausal state in women is accompanied by a higher risk of metabolic and CVDs (Polotsky and Polotsky 2010; El Khoudary et al. 2020; Ding et al. 2022). This phenomenon is caused by the

changes in sex hormone levels in the menopausal transition period, where endogenous estrogen levels decrease (Bittner 2009; Neff et al. 2022). Moreover, atherosclerosis, increased epicardial fat, and endothelial dysfunction are observed during this transition state (Matthews et al. 2009; Cabrera-Rego et al. 2018; Everson-Rose et al. 2021). It is believed that PFAS exposures play a role in further developing CVDs in women during the menopausal transition (Wang et al. 2021; Ding et al. 2022; Rickard et al. 2022). Still, the biological mechanism of how PFAS affects the cardiovascular system during postmenopausal state is not well understood.

In this study, we hypothesize that exposure to PFAS in our cardiovascular cohort study will have a distinct biomarker signature. We explored the association between 2 legacy long-chain PFAS that have been phased out of production in some countries, perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), and a short-chain emerging PFAS, perfluorobutanesulfonic acid (PFBS), which was introduced as an alternative to older phased out PFAS in postmenopausal women using metabolomic and proteomics analyses. Along with machine learning techniques, to help us predict a pattern between the PFAS detected and our cardiovascular cohort study, our findings provide new insight into the potential mechanism underlying the increased risk of cardiovascular events in postmenopausal women and the emerging evidence on PFAS-induced risk of CVDs in this population.

1. Materials and methods

1.1 Study design and population

All the samples were handled and analyzed under Izmir Katip Celebi University Interventional Clinical Studies Institutional Review Board IRB Protocol No. 80. This prospective observational study involved 70 patients in total from Turkey, between the ages of 45 to 78 yrs. Among them, 23 were identified with coronary microvascular disease (CMD), 21 with coronary artery disease (CAD), and 26 constituted the control group. The inclusion criteria for this study included chest pain CAD-related, positive noninvasive imaging results (cardiovascular stress test, myocardial perfusion scintigraphy), and an effective coronary angiography (CAG) intervention. The exclusion criteria included being male, premenopausal female, patients who refused to participate in the study, and having a contraindication against CAG. CAG was done using the Judkins technique through the femoral or radial artery. Patients were provided with the information about the study and their consent was obtained. For patients who were found to have CAD or CMD after CAG intervention, we obtained 5 ml of blood. The control group had medical and demographic characteristics comparable to those of our other cardiovascular group patients. Further patient characteristics and study details were previously described (Arredondo Eve et al. 2021).

1.2 Chemical compounds

For plasma samples, 3 PFAS were examined: PFOA ($C_8HF_{15}O_2$), PFOS ($C_8HF_{17}O_3S$), and PFBS ($C_4HF_9O_3S$). As internal standard (IS), perfluorodecanoic acid (PFDA, purchased from Sigma—177741) was used.

1.3 Targeted PFAS analysis

For validation and quality control of PFAS compounds (PFOA, PFOS, PFBS) in biological samples, limit of quantification (LOQ) was done. Liquid chromatography with tandem mass spectrometry (LC-MS-MS) analysis was performed at UIUC Metabolomics Center. Briefly, 50 µl of blood plasma was mixed with 150 µl of pure methanol and 30 µl of the IS (PFDA), vortexed, and centrifuged for 10 min at 14,000 rpm at 4°C. The exact amount of IS was spiked with standard solutions. PFAS compounds (PFOA, PFOS, PFBS) were measured with Agilent Technologies 1290 Infinity Series (Agilent Technologies, Santa Clara, CA, United States) HPLC and a SCIEX 5500 QTRAP mass spectrometer (AB SCIEX Technologies, Framingham, MA, United States) in electrospray ionization negative MRM mode. The analytical column was an Eclipse Plus C18, 3.5 µm, 100 × 4.6 mm (Agilent, USA). The eluents were methanol (mobile phase B) and LC-MS grade water containing 10 mM ammonium formate (mobile phase A). The column flow rate was 350 µl/min. Peak integration and quantitation were done using Analyst (V. 1.7.1) software.

1.4 Plasma metabolite detection and quantification

To identify and quantify the metabolites in plasma, a gas chromatography-mass spectrometry (GC/MS) analysis was performed at UIUC Metabolomics Center as described (Palazoglu and Fiehn 2009; Madak-Erdogan et al. 2019; Eve et al. 2020; Oktay et al. 2020; Smith et al. 2020). Briefly, we extracted 50 µl of blood plasma using 1 ml of isopropanol: acetonitrile: water (3:3:2, v/v) at 20°C for 5 min. After centrifugation, we dried the 0.5 ml of supernatant in a SpeedVac concentrator and subsequently derivatized in 2 steps: with 50 µl methoxyamine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) (40 mg/ml in pyridine) for 60 min at 50°C, then with 50 µl MSTFA + 1% TMCS (Thermo, Waltham, MA, United States) at 70°C for 120 min, followed by a 2-h incubation at room temperature. Thirty microliters of 1 mg/ml Hentriacontanoic acid were added to each sample before derivatization for use as an IS for normalization. A gas-chromatography mass-spectrometry (GC–MS) system (Agilent Inc, Santa Clara, CA, United States) was used to detect the metabolites consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 MSD and 7683 B autosampler, as previously described (Borgogna et al. 2020). The scan range was set at least 50 m/z above the highest anticipated fragment. The minimum quality match for minor compounds was ≥80 and for other peaks ≥90. The spectra of all chromatogram peaks were evaluated using the AMDIS 2.71 (NIST, Gaithersburg, MD, United States) using a custom-built MS database (484 unique metabolites) (Kerner and Hoppel 2000). All known artificial peaks were identified and removed before data mining. To allow comparison between samples, all data were normalized to the IS in each chromatogram.

1.5 Plasma protein detection and quantification

Fifty microliters of EDTA plasma were used for Plasma OLINK Proseek, target 96 cardiovascular panels II and III (CVD II and CVD III) proximity extension assay (PEA) proteomics, Uppsala, Sweden. OLINK Proseek Multiplex technology is based on a proximity extension assay that uses a matching pair of antibodies linked to unique oligonucleotides to detect and quantify predefined biomarkers. Values are given as normalized protein expression and are log2 transformed as described in detail elsewhere (Lind et al. 2015; Petrera et al. 2021). Olink target 96 cardiovascular panels has a comprehensive selection of proteins associated with biological functions linked to CVDs. This categorization was done using public access databases, including Uniprot, Human Protein Atlas, Gene Ontology (GO), and DisGeNET. Both CVD II and CVD III measure 184 proteins known to be human cardiovascular and inflammatory markers as well as some exploratory human proteins with the potential to be new CVD markers.

1.6 Machine learning analysis: data preprocessing, feature selection, and classification

The raw data consisting of relative concentration of 3 target compounds (PFOA, PFOS, PFBS) measured across 70 patients was cleaned; missing values were imputed, and the columns with missing values were removed. For the entire process of data cleaning, data preprocessing, data analysis, and the performing of various algorithms, R programming language was used. Boruta, caret, tidyverse, readxl, e1071, pROC, tree, and random forest (RF) were also utilized for the ensuing analysis. We employed principal component analysis , but it lacked dimensionality reduction; therefore, feature selection algorithms, including boruta test, recursive feature elimination (RFE), simulated annealing, and genetic algorithm were performed. To calculate important features in RFE, we used "repeated cross-validation" method with repeats = 5. After obtaining important features, we performed logistic regression, RF, decision trees (DTs), and support vector machines (SVMs). Receiver operating curves for all the methods were plotted, and sensitivity, specificity, precision, recall, and F1 score were calculated using the confusion matrix through true positives, true negatives, false positives, and false negatives.

1.7 Pathway analysis

To identify the metabolic pathways related to our PFAS and our study groups, a pathway analysis was performed using Metaboanalyst 4.0 pathway analysis software (https://www.metaboanalyst.ca), which combines pathway enrichment analysis with pathway topology analysis (Xia et al. 2009; Lu et al. 2023). Metaboanalyst 4.0 utilizes the Kyoto Encyclopedia of Genes and Genomes metabolic pathways as its foundational knowledge base, but it also integrates well-known methods such as univariate analysis, overrepresentation analysis, along with innovative algorithms and concepts test, and network topology analysis into the path analysis. This analysis was limited to all metabolic features predicted by RF, which also significantly correlated to the specific PFAS and the disease status (as predicted by machine learning method).

1.8 Protein–protein interaction analysis

To investigate the relationship between the protein network structure and function, all proteins that were significantly correlated with PFOA, PFOS, and PFBS in our CVD groups (CMD, CAD) were included in a protein–protein interaction analysis done using STRING v12.0 (https://string-db.org) software (Franceschini et al. 2016; Szklarczyk et al. 2019, 2023) using a minimum required interaction score of high confidence (0.700) as a limit. STRING software is a database where identified protein–protein connections are well established based on data from automated text mining of scientific literature, computational interaction predictions from coexpression, conserved genomic context, databases of interaction experiments, and known complexes/pathways from curated sources, and utilized in many studies for protein connections and function (Dunder et al. 2023; Li et al. 2023; Szklarczyk et al. 2023). In STRING, we also used reactome pathway analysis, which establishes systematic connections between proteins (in our case, human proteins) and their molecular functions, serving as both a repository for biological processes and as a valuable way for uncovering new functional relationships in datasets in a single data model (Fabregat et al. 2017; Sidiropoulos et al. 2017).

1.9 Statistical methods

An unpaired *t*-test was performed to compare PFAS level differences between groups. Geometric means, selected percentiles (50th, 75th, 90th, 95th), and distribution-free 95% CIs of serum PFAS levels among our cardiovascular study participants were estimated using proc survey means. These estimates were compared with the national averages and percentiles from the 2017 to 2018 National Health and Nutrition Examination Survey (NHANES). Comparison for PFBS level was conducted with 2013 to 2014 NHANES data because PFBS information was not available in the 2017 to 2018 NHANES data. Correlation analysis using Pearson r correlation and simple linear regression was performed for metabolite and protein levels of our cardiovascular groups and PFOA, PFOS, and PFBS levels. All statistical analyses were performed using SAS v 9.4 (SAS Institute, Carry, NC) and GraphPad Prism 9.3.1 (GraphPad Software Inc., La Jolla, United States), and a *P*-value of <0.05 was considered statistically significant.

2. Results

2.1 Participant's characteristics and group classification

Participant's characteristics and group classification are detailed in a previous study (Arredondo Eve et al. 2021).

2.2 Quality control for PFAS detection

Validation and quality control of PFAS compounds in our biological samples were done using limit of quantification (Tables 1 and 2). This analysis detected PFOA (5 pM), PFOS (20 pM), and PFBS

Table 1. Compound recovery from biological sample using PFDA (IS).

	IS peak area	
	IS, extract	**IS, methanol**
	7.57E + 06	1.26E + 07
	9.85E + 06	1.30E + 07
	8.99E + 06	1.29E + 07
	1.50E + 07	1.34E + 07
Average	1.04E + 07	1.30E + 07

Abbreviations: IS, internal standard; PFDA, perfluorodecanoic acid.

Table 2. Levels of concentration of internal standard (IS) in biological samples on different concentration in 3 days (rounds).

Day 1			Day 2			Day 3	
Conc (nM)	**nM**	**Accuracy, %**	**Conc (nM)**	**nM**	**Accuracy, %**	**Conc (nM)**	**nM**
0.01	0.009	85.35	0.01	0.0123	93.5	0.01	<0
0.01	0.014		0.01	<0		0.01	<0
0.01	0.012		0.01	<0		0.01	<0
0.01	0.010		0.01	0.009		0.01	<0
10	8.6	95.15	10	8.9	87.88	10	1.0
10	9.1		10	8.4		10	1.2
10	10.0		10	8.7		10	1.9
10	10.3		10	9.1		10	2.7
100	93.6	99.63	100	87.5	88.25	100	26.4
100	99.4		100	86.2		100	44.5
100	99.5		100	88.7		100	55.6
100	106		100	90.6		100	50.0

On day 3 targets compounds decomposed being 72 h at 10°C.
Abbreviation: Conc, concentration.

(5 pM), respectively. Since all 3 target compounds were found in our biological sample extracts, compound recovery was assessed using an IS with similar properties and recovery as our target PFAS compounds. The recovery was almost 80%, so we assume all target compounds were extracted from samples similarly. IS was added to our samples and assessed in 3 rounds (days 1 to 3), with 3 levels of concentration (1.01 nM, 10 nM, 100 nM). Day 1, 1.01 nM (accuracy of 85.35%), 10 nM (accuracy of 95.15%), 100 nM (accuracy of 99.63%). Day 2, 1.01 nM (accuracy of 93.5%), 10 nM (accuracy of 87.88%), 100 nM (accuracy of 88.25%). Day 3 targets compounds decomposed being 72 h at 10°C.

2.3 PFAS serum concentration comparison between CVD study and NHANES

Comparison of our CVD study PFOA, PFOS, and PFBS serum concentration to the 2011 to 2018 National Health and Nutrition Examination Survey (NHANES) serum concentration (Fig. 1) showed that PFBS levels are much higher in our samples than in NHANES. In contrast, PFOA is higher in NHANES studies when compared with our CVD studies, but PFOS levels we comparable in both (Table S1).

2.4 PFOS is a good predictor of CAD, whereas PFOA is an intermediate predictor of CMD

To further assess if exposure to PFAS is associated with CVD in postmenopausal women, we applied a methodology that combines traditional statistical modeling with machine learning methods to perform predictor selection and ranking. We evaluated features that contribute to control versus CAD and control versus CMD. Diverse classification methods (logistic regression, DT, RF, and SVM) were used to assess which feature is common in this classification (Table S2). The classification performance results showed that different selection methods were successful in examining the relationship between CAD and CMD groups for PFOS only, PFOA only, or PFOA/PFOS (Table S2). Control versus CAD had moderate accuracy in the RF classification method for CAD and PFOS only, with an area under the curve (AUC) of 78% (Fig. 2A). In contrast, Control versus CMD group, classification method combinations had lower to intermediate accuracy for PFOA only and PFOS only; all AUCs were below 65% (Fig. 2B).

2.5 PFAS levels are associated with altered abundance of proteins linked to inflammation

Overall, 31 proteins were significantly correlated to the different PFAS detected in our CVD group's blood samples. For PFOA (Table S3), CMD disease group has 2 proteins significantly correlated: Interleukin-18 (IL-18) and low-affinity immunoglobulin gamma Fc region receptor II-b (IgG Fc receptor II-b); CAD group has 5 proteins correlated: Thrombospondin-2 (THBS2), gastrotropin (GT), integrin beta-2 (ITGB2), granulins (GRN), and retinoic acid receptor responder protein 2 (RARRES2).

PFOS concentrations were associated with the highest number of proteomic biomarkers in our CVD groups (Table S4). For CMD, a total of 6 proteins were significantly associated with PFOS: Alpha-L-iduronidase (IDUA), matrix metalloproteinase-7 (MMP7), IgG Fc receptor II-b, protein-glutamine gamma-glutamyl transferase 2 (TGM2), myeloblastin (PRTN3), and C-C motif chemokine 16 (CCL16). For CAD disease group, a total of 13 proteins were

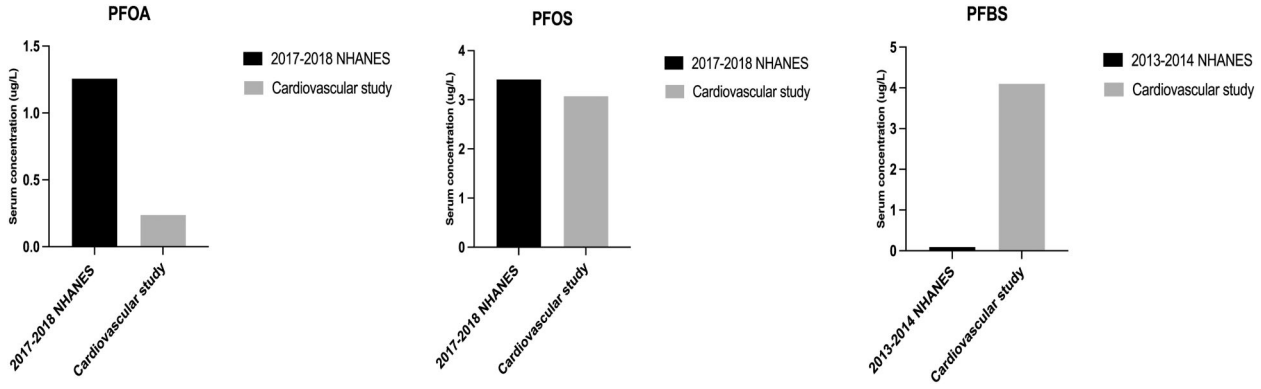

Fig. 1. Comparison of PFAS values from CVD cohort and National Health and Nutrition Examination Survey (NHANES). PFOA, PFOS (NHANES table: female year 2017 to 2018), PFBS (NHANES table: female year 2013 to 2014); PFBS levels were below <LOD.

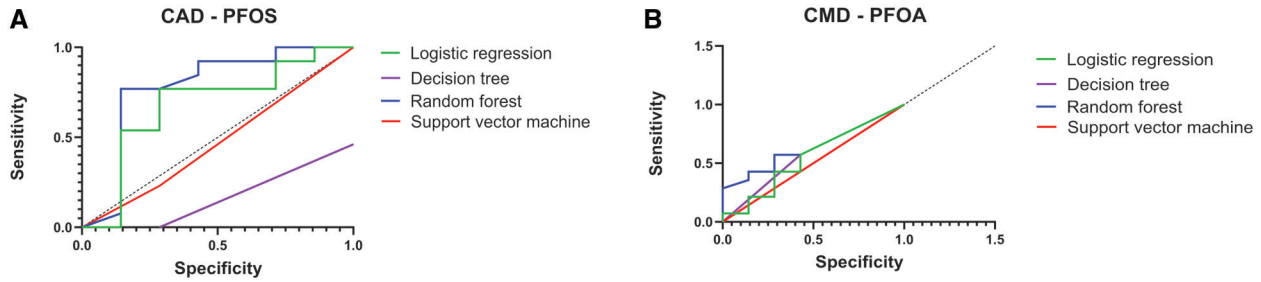

Fig. 2. Comparison classification method for PFAS association with disease status. A) Coronary artery disease (CAD), B) coronary microvascular disease (CMD).

correlated significantly to PFOS: Interleukin-4 receptor subunit alpha (IL-4RA), gastric intrinsic factor (GIF), growth hormone (GH), tyrosine-protein kinase Mer (MERTK), carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8), C-C motif chemokine 3 (CCL3), gastrotropin (GT), cathepsin L1 (CTSL1), fatty acid-binding protein adipocyte (FABP4), azurocidin (AZU1), myeloperoxidase (MPO), interleukin-1 receptor type 2 (IL-1RT2), and matrix extracellular phosphoglycoprotein (MEPE).

Finally, for PFBS (Table S5), CMD group has only 2 proteins significantly correlated: TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) and elafin (PI3), but for CAD group, 3 proteins were significantly correlated: Heme oxygenase 1 (HO-1), insulin-like growth factor-binding protein 1 (IGFBP-1), and collagen alpha-1(I) chain (COL1A1).

To visualize the biological connection between the significant protein of each PFAS and disease group described above, STRING database was utilized. A high confidence interaction score of > 0.700 was selected, and Markov cluster algorithm of 3 was used, as described in previous study (Dunder et al. 2023). In Figs S1 to S3, protein interaction network shows up to 5 groups of related protein clusters among the different PFAS and disease groups. Protein biological function from STRING is shown in Tables S6 to S11.

Next, to provide a better understanding of these connections, common reactome pathways associated with our proteins were used and envisioned in Figs 3 and 4, but the complete reactome pathway analysis is shown in Tables S12 to S14. The 5 most enriched pathways are described as follows: Type of PFAS—disease group—pathway name—number of proteins involved. For PFOA-CMD (Fig. 3A), the immune system (n = 12), signaling by interleukins (n = 8), interleukin-1 family signaling (n = 7), innate immune system (n = 5), interleukin-4, and interleukin-13 signaling (n = 3) pathway are involved. For PFOS-CMD (Fig. 3B), the immune system (n = 12), signaling by interleukins (n = 6), neutrophil degranulation (n = 6), degradation of the extracellular matrix (n = 4), the innate immune system (n = 7) pathways are associated. For PFBS-CMD (Fig. 3C), signal transduction (n = 11), death receptor signaling (n = 10), apoptosis (n = 10), the immune system (n = 7), and disease (n = 6) pathways are involved.

For PFOA-CAD (Fig. 4A), the immune system (n = 10), innate immune system (n = 8), immunoregulatory interactions between a lymphoid and a nonlymphoid cell (n = 7), neutrophil degranulation (n = 6), and hemostasis (n = 5) pathways are associated. For PFOS-CAD (Fig. 4B), the immune system (n = 17), signaling by interleukins (n = 11), cytokines signaling in the immune system (n = 12), innate immune system (n = 9), and neutrophil degranulation (n = 6) pathways are involved. For PFBS-CAD (Fig. 4C), signaling by receptor tyrosine kinases (n = 8), degradation of the extracellular matrix (n = 6), hemostasis (n = 5), platelet activation, signaling, and aggregation (n = 4), and vesicle-mediated transport (n = 4) pathways are associated.

2.6 PFAS-CVD-specific metabolic signatures

We analyze the correlation of PFAS levels with the metabolites and protein levels in our cardiovascular study. For all 3 cardiovascular study groups (control, CMD, and CAD) and all 3 PFAS (PFOA, PFOS, and PFBS), a unique metabolic pattern existed (Fig. 5). The specific names of the metabolites and proteins with significant correlation between the different PFAS and our cardiovascular groups are vast and are shown in Tables S3 to S5.

Using the information from our prediction model, we performed metabolic pathway analysis for the metabolites and proteins of CMD group that were significantly correlated with PFOA and for PFOS and CAD group. We observed that the correlated metabolites and proteins in PFOA and CMD mainly involved biosynthesis, metabolism, and degradation of amino acids (AA) (Fig. 6A–C). In contrast, PFOS and CAD correlated metabolites and proteins are involved not only in AA biosynthesis, metabolism, and degradation but also in glycolysis and gluconeogenesis, peroxisome proliferator-activated receptor (PPAR) signaling pathway, among others (Fig. 6D–F).

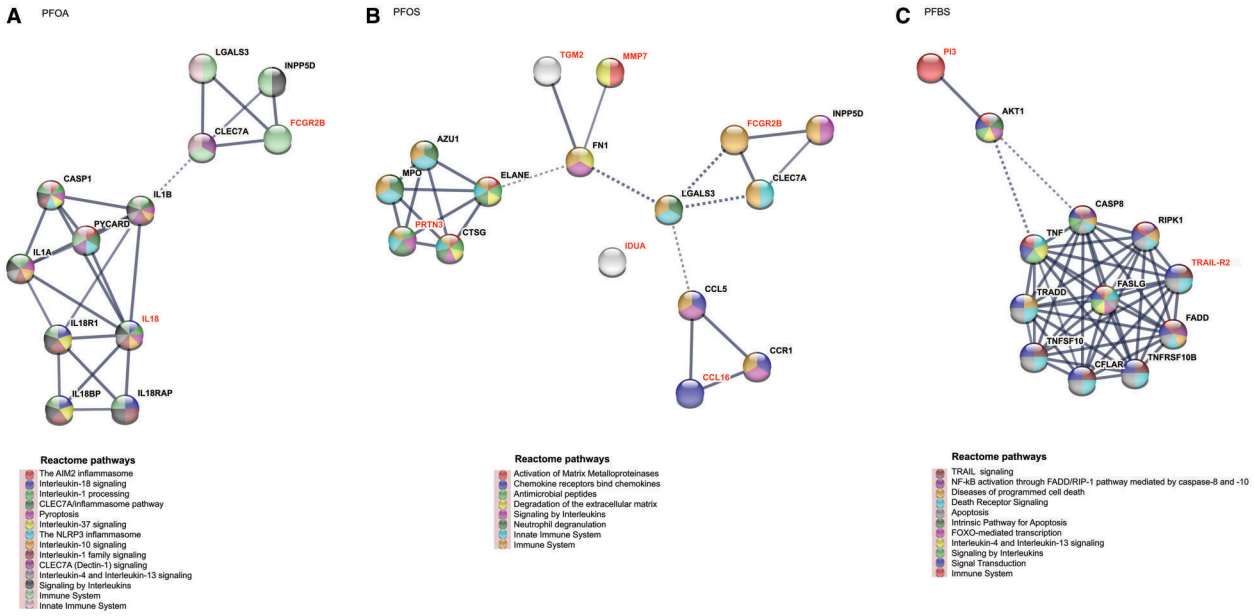

Fig. 3. Reactome pathway analysis of protein-to-protein interaction associated with CMD. A) PFOA, B) PFOS, C) PFBS. Disease group proteins that had a significant association with PFAS and that meet a minimum high confidence interaction score of >0.700 are included. The edges between the protein nodes are proportional to the node indicates the reactome pathway with which the protein is associated.

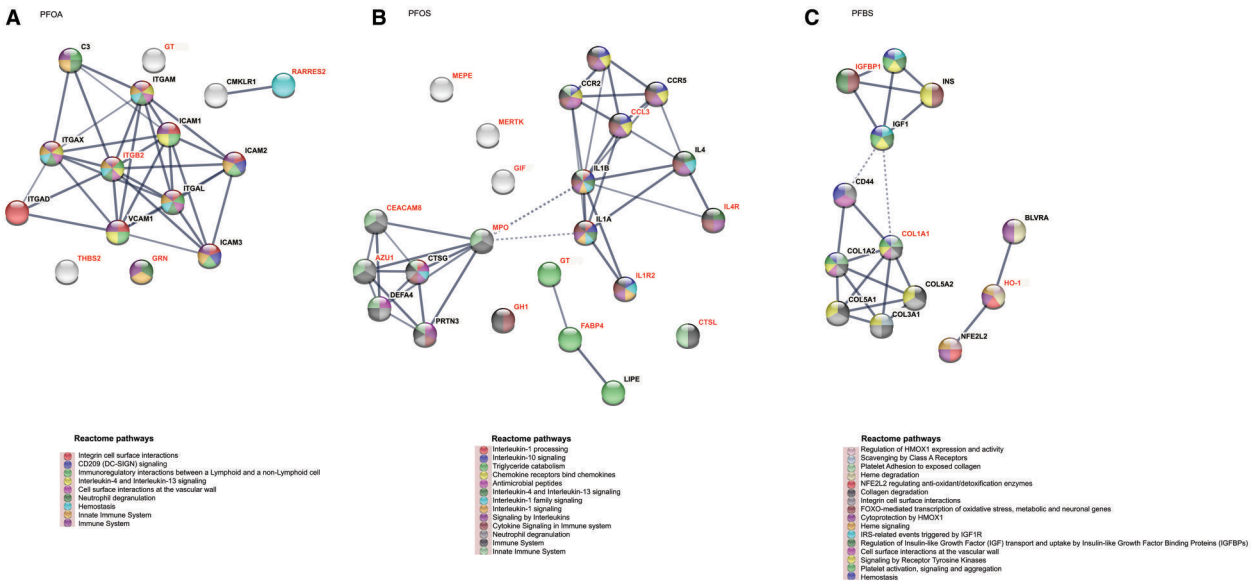

Fig. 4. Reactome pathway analysis of protein-to-protein interaction associated with CAD. A) PFOA, B) PFOS, C) PFBS. Disease group proteins that had a significant association with PFAS and that meet a minimum high confidence interaction score of >0.700 are included. The edges between the protein nodes are proportional to the node indicates the reactome pathway with which the protein is associated.

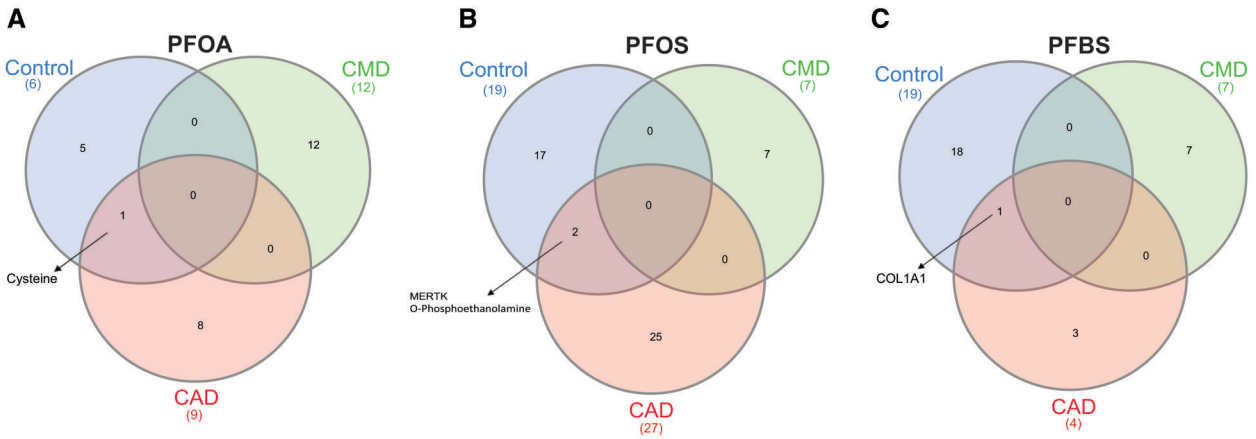

Fig. 5. Venn diagram of metabolites and proteins significantly correlated to A) PFOA, B) PFOS, and C) PFBS in our cardiovascular cohort groups.

3. Discussion

In the current study, we reported an association between PFAS, cytokine, and metabolite levels in our cardiovascular group study. The PFAS levels correlated positively or negatively with concentrations of metabolites and proteins belonging to diverse metabolic pathways. These findings suggest that different PFAS can alter the level of proteins or metabolites belonging to similar metabolic pathways in postmenopausal women.

We found that participants from all 3 groups (control, CMD, CAD) have PFOA, PFOS, and PFBS levels detected in their blood, consistent with earlier studies (Kärrman et al. 2006; Frigerio et al. 2022). No statistical significance was found between our cardiovascular groups and PFAS levels, but when compared with NHANES study, which aims to monitor the health and nutritional status of the US population and is used as a guide of comparison in environmental research (Tian et al. 2022; Wen et al. 2022); it was observed that PFOS levels in our CVD study were similar to NHANES levels, PFBS levels in our CVD study were far higher than NHANES–PFBS levels in NHANES was below the level of detection (LOD); in contrast, our levels of PFOA were lower than NHANES levels (Fig. 1). In the United States, PFOS and PFOA were banned in 2002, and PFBS has been used as a replacement PFAS since 2003. The ban of PFOA took effect earlier in 2019, highlighting the importance of decreased exposure to reduce levels in the body (Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological Profiles 2021). However, higher levels of PFBS are concerning as the emerging compounds might be toxic and persistent in the body (Huang et al. 2019; Liu et al. 2020, 2022; Hamid et al. 2023). Overall, these findings show that our samples are comparable to the US female population exposed to PFAS.

Next, we used the machine-learning algorithms to identify if there were any PFAS that classified CVD groups. Previously, we used these approaches for identifying breast cancer risk biomarkers (Oktay et al. 2020; SHu et al. 2022; Ferre et al. 2023; Nazari et al. 2023), metabolic indicators of breast cancer disparities (Santaliz-Casiano et al. 2023), liver carcinogenesis gene markers (Smith et al. 2020), and health indicators of food consumption (Eve et al. 2020). For the CAD group of our CVD study,

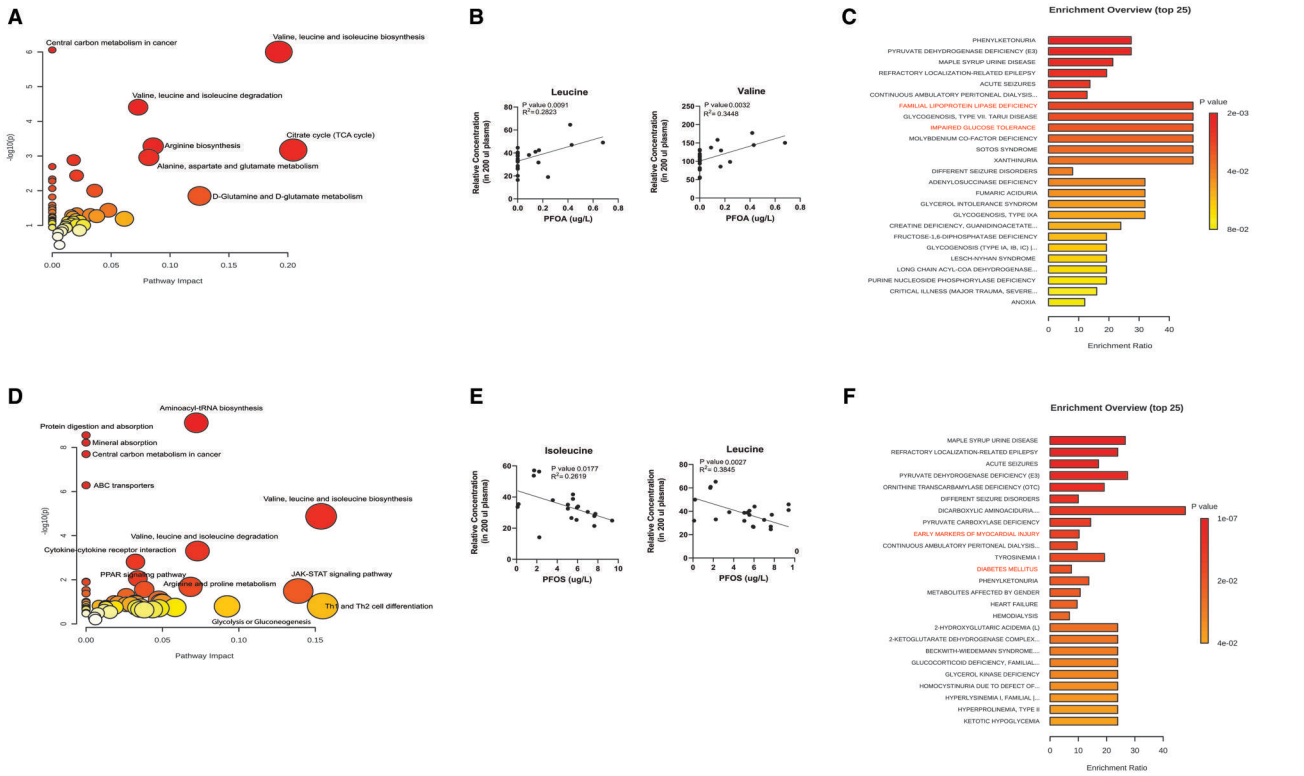

Fig. 6. Integrated pathway analysis, linear correlation, and metabolites disease signature of metabolites and proteins of A–C) PFOA and CMD and D–F) PFOS and CAD groups.

PFOS was the selected feature supported by high selectivity and specificity with high mean AUC values when selection methods and classification were performed (Fig. 2A). The AUC was 78% using RF as the classification method—an acceptable marker of increased risk of cardiovascular artery disease when postmenopausal women are exposed to PFOS (Akobeng 2007; Mandrekar 2010; Nahm 2022). In the case of the CMD group, PFOA had the best classification performance when RF approach was used as a classification method, which reported an AUC value of 63% (Fig. 2B) (Akobeng 2007; Mandrekar 2010; Nahm 2022). These findings suggest that in postmenopausal women, the risk of a specific type of CVD might be affected by the type and level of PFAS that they are exposed to.

Since we observed an association between PFAS levels in different CVD groups, we analyzed the relationship between the PFAS levels (PFOA, PFOS, and PFBS) identified in our CVD study and the metabolites and protein levels of each of our groups to help us unravel which biological pathways are affected by the exposure of these PFAS. Our analyses showed a distinct pattern of metabolites and proteins for each group and each PFAS studied (Fig. 5). Further analysis based on our machine learning results, it was observed that CMD metabolites were all positively correlated to PFOA (Table S3); in contrast, in CAD, all the significant metabolites were negatively correlated to PFOS (Table S4). These findings support the idea that exposure to PFAS in postmenopausal women has the potential to impact heart disease outcomes, affecting the same metabolic pathways in different ways.

We use protein-to-protein network analysis to visualize the functional relationship and interaction between the significant proteins in our CVD group and our different PFAS. Figures S1 to S3, show all 3 PFAS and CVD group's related proteins are clustered differently, showing their relation and how they may or may not be interconnected. We also performed reactome pathway analysis, where it was observed that most of the proteins involved in both diseases and the PFAS studied play a role in the proinflammatory signaling (Figs 3 and 4). These findings help us understand how these PFAS can affect the cardiovascular system and increase the risk of CVD in this population.

When integrated pathway analysis of the metabolites and proteins that were significantly correlated in CAD/PFOS and CMD/PFOA was performed, it was found that for both diseases and both PFAS, related pathways are affected (Fig. 6B, C, E, and F). Interestingly, AA biosynthesis, metabolism, and degradation pathway, especially levels of valine and leucine, are affected when both CMD/PFOA and CAD/PFOS correlations were done, but with an opposite relationship. Metabolite disease signature analysis (Fig. 6C and F) showed that metabolic diseases such as familial lipoprotein lipase deficiency, and impaired glucose tolerance were identified in CMD/PFOA correlation group. Several pathways including early markers of myocardial injury, and diabetes mellitus were affected in CAD/PFOS correlation analysis. These findings indicate that regardless of the type of CVD, similar biological pathways are altered by different PFAS, suggesting a common biological pathway response to PFAS presence.

CAD is an inflammatory disorder (Ross 1999; Libby and Theroux 2005; Malakar et al. 2019) that can be expressed as stable angina, unstable angina, myocardial infarction, or sudden cardiac death (Rahimtoola 1995; Braunwald et al. 2002). It is one of the most common types of heart disease in the United States (Cooper et al. 2000; Odden et al. 2011; Duggan et al. 2022) and can be caused by environmental or genetic factors (Khera and Kathiresan 2017; Malakar et al. 2019; Akyuz 2020). Lifestyle has been found to play a vital role in the development of such CVDs (Bauersachs et al. 2019; Akyuz 2020; Duggan et al. 2022). CAD occurs due to atheroma plaque that can occlude over time in the coronary arteries of the heart, affecting the endothelial function

of the arterial wall (Ross 1999; Akyuz 2020; Fox et al. 2020). Some risk factors associated with the development of CAD are smoking, diabetes, hyperlipidemia, hypertension, being a postmenopausal woman, and men >45 yrs old (Bauersachs et al. 2019; Akyuz 2020; Fox et al. 2020; Duggan et al. 2022). We identified metabolites, such as AAs, that are involved in the myocardial adaptation (Wang et al. 2016; Li et al. 2020; Xuan et al. 2021) and proteins that serve as proinflammatory cytokines or are part of the inflammatory process to correlate with PFOS levels, which might be related to the disease pathogenesis. Our integrated pathway analysis of the metabolites and proteins showed that PFOS/CAD was related to diseases such as diabetes mellitus, where CAD has been described as one of the main causes of mortality in diabetes mellitus (Aronson and Edelman 2014; Ofstad 2016; Chakraborty et al. 2023). Overall, our findings support a role for PFAS contributing to CAD pathophysiology by modulating levels of biomolecules relevant to this disease.

CMD is the most common cause of cardiac ischemic chest pain in patients without obstructive CAD (Haasenritter et al. 2015; Laureano-Phillips et al. 2019; Mileva et al. 2022). The reasons for CMD can be heterogeneous, and the possible mechanisms include endothelial and smooth muscle dysfunction, inappropriate sympathetic tone, microvascular atherosclerosis, and inflammation (Crea et al. 2014; Corcoran et al. 2018; Lanza et al. 2022). We found that PFOA correlated with an increase in metabolites such as valine and leucine in this group. Branched-chain AAs such as valine, leucine/isoleucine, glutamate/glutamine, proline, and methionine are associated with and are predictive of clinical events in patients with CVD (Shah et al. 2010; Xuan et al. 2021); we also observed that impaired glucose tolerance was related to PFOA/CMD in our integrated pathway analysis of metabolic disease signature, which can predispose to cardiovascular alterations over time if left untreated (Ceriello 2004; Schnell and Standl 2006). Our results suggest that PFAS may play a part in the physiological process associated with CMD, particularly by increasing inflammatory signaling that was shown to increase microvascular dysfunction (Tunc et al. 2020; Godo et al. 2021a, 2021b). Of note, in our previous study, we noted a role for increased free fatty acid levels (Arredondo Eve et al. 2021). PFAS are structurally similar to free fatty acids and was shown to work through PPARs (Imir et al. 2021; Boyd et al. 2022; Hu et al. 2022; Santaliz Casiano et al. 2022). Increased PFAS might levels might activate these receptors in the cardiovascular system (Hamblin et al. 2009). Further studies are needed to definitively show PFAS-mediated activation of inflammation and PPAR signaling to increase CMD etiology.

Previous studies on PFAS and the cardiovascular system support our proteomic findings. In a middle-aged population, it was found that PFOA, PFOS, and perfluorohexane sulfonate altered the levels of proteins linked to inflammation, metabolism, and CVDs (Dunder et al. 2023)—similar to our findings. In another study, a systematic evidence map (SEM) was done to search for the relationship between PFAS, chronic inflammation, immunosuppression, and cancer based on the International Agency for Research of Cancer and the National Toxicology Program. It was confirmed that the PFAS studied might induce immunosuppression and chronic inflammation (Zhang et al. 2023), 2 of the most common pathways affected in our legacy PFAS and our CVDs, when reactome pathway analysis was done. We also observe similar changes when comparing our metabolic pathway findings with other studies. A study used pregnant women's and children's blood samples to measure PFAS and metabolomic profiling. It was observed that metabolites related to lipid metabolic pathways and AAs, such as leucine, isoleucine, valine, and methionine, were affected in response to PFAS exposure (Prince et al. 2023). Another study assessed metabolite and metabolic pathway alterations associated with PFAS exposure; it concluded that the pathways related to lipids and AAs were the most reported associated with PFAS exposures (Guo et al. 2022).

This study has strengths and limitations, including the small number of samples per group and the origin of participants not from the United States. We compared our data to NHANES, which has a similar analysis in participants from the United States; for future studies, we will need to validate these findings with a higher number of participants from the United States, in postmenopausal participants, and not the female-only population. Nonetheless, this study provides a broad metabolic assessment of pathways associated with PFAS exposure and the risk of CVD, utilizing multiple data analysis methods. Also, only a few previous studies have investigated the effects of PFAS exposure on metabolic and proteomic biomarkers in CVDs related to postmenopausal women. Further, we initially selected PFOA and PFOS because these are the legacy compounds and are still detectable in human blood. PFBS was selected because this compound replaced PFOA and PFOS after the ban of use of these chemicals in 2002. Since this analysis, our analytic capabilities to detect different legacy and emerging PFAS increased. Our findings with these 3 compounds will provide the basis for future studies to examine levels and CVD association of different PFAS.

In conclusion, our data suggests an association between PFAS exposure and changes in the abundance of proteins and metabolites that might point out to abnormalities in the health of the cardiovascular system in postmenopausal women. Overall, exposure to PFOA and PFOS chemicals can affect metabolic pathways that might increase the risk of CMD and CAD in postmenopausal women utilizing the same metabolic pathway but in different ways or utilizing a completely different metabolic pathway.

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Supplementary material

Supplementary material is available at *Toxicological Sciences* online.

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Conflicts of interest

None declared.

Institutional review board statement

Studies are approved by the Izmir Katip Celebi University Interventional Clinical Studies Institutional Review Board (IRB

No. 80). All research was carried out in compliance with the Helsinki Declaration.

Informed consent statement

Donors provided broad written consent for using their specimens in research. The consent document informed the donor that the donated specimens and medical data would be used for the general purpose of helping to determine biomarkers of CMD in postmenopausal women.

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