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Non-targeted metabolomics and associations with per- and polyfluoroalkyl substances (PFAS) exposure in humans: A scoping review

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Abstract

Objective—To summarize the application of non-targeted metabolomics in epidemiological studies that assessed metabolite and metabolic pathway alterations associated with per- and polyfluoroalkyl substances (PFAS) exposure.

Recent Findings—Eleven human studies published before April 1st, 2021 were identified through database searches (PubMed, Dimensions, Web of Science Core Collection, Embase, Scopus), and citation chaining (Citationchaser). The sample sizes of these studies ranged from 40-965, involving children and adolescents (n=3), non-pregnant adults (n=5), or pregnant women (n=3). High-resolution liquid chromatography–mass spectrometry was the primary analytical platform to measure both PFAS and metabolome. PFAS were measured in either plasma (n=6) or serum (n=5), while metabolomic profiles were assessed using plasma (n=6), serum (n=4), or urine (n=1). Four types of PFAS (perfluorooctane sulfonate (n=11), perfluorooctanoic acid (n=10), perfluorohexan sulfonate (n=9), perfluorononanoic acid (n=5)) and PFAS mixtures (n=7) were the most studied. We found that alterations to tryptophan metabolism and the urea cycle were most reported PFAS-associated metabolomic signatures. Numerous lipid metabolites were also

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suggested to be associated with PFAS exposure, especially key metabolites in glycerophospholipid metabolism which is critical for biological membrane functions, and fatty acids and carnitines which are relevant to the energy supply pathway of fatty acid oxidation. Other important metabolome changes reported included the tricarboxylic acid (TCA) cycle regarding energy generation and purine and pyrimidine metabolism in cellular energy systems.

Conclusions—There is growing interest in using non-targeted metabolomics to study the human physiological changes associated with PFAS exposure. Multiple PFAS were reported to be associated with alterations in amino acid and lipid metabolism, but these results are driven by one predominant type of pathway analysis thus require further confirmation. Standardizing research methods and reporting are recommended to facilitate result comparison. Future studies should consider potential differences in study methodology, use of prospective design, and confounding bias and measurement errors.

Keywords

Exposome; Metabolomics; Persistent Organic Pollutants; Perfluorinated Compounds; Polyfluoroalkyl Substances

1. Introduction

Recent developments in mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have allowed for a comprehensive and quantitative high-resolution phenotyping of non-targeted metabolic alterations at the molecular level (Holmes et al. 2008; Hu et al. 2020; Jones et al. 2012). This advanced non-targeted workflow can comprehensively and simultaneously assess hundreds or thousands of exogenous chemicals, their metabolites, and associated endogenous metabolic perturbations in small volumes of biological samples (Jin et al. 2021; Tzoulaki et al. 2014). Pathway-mapping and network modeling can further place identified metabolite features into interconnected biological pathways and into context with upstream genes and proteins (Johnson et al. 2016). Furthermore, the characterization of metabolic fingerprints and the deciphering of pathways can be linked to disease risk factors in the general population. This approach is known as the metabolome-wide association study (MWAS), a conceptual and technological tool to reveal the complex cellular mechanisms underlying environmentally mediated diseases. Briefly, the metabolome markers could be important intermediates to investigate the intricate triple relationship between exposure, molecular effect, and clinical outcomes (Bictash et al. 2010; Cai et al. 2020; Chadeau-Hyam et al. 2011; Lu et al. 2019; Nicholson et al. 2008; Rattray et al. 2018).

There has been increasing concern with respect to the potential health effects of per- and polyfluoroalkyl substances (PFAS) (Giesy and Kannan 2001; Sunderland et al. 2019). PFAS are a group of fluorinated chemicals widely used in industrial and commercial applications, including kitchenware, food packaging, clothing, carpeting and coating (Houde et al. 2006; Lau 2015; Wang et al. 2017b). PFAS are persistent and ubiquitous in the environment, and frequently detected in populations globally (Bjerregaard-Olesen et al. 2016; Calafat et al. 2019; Lau et al. 2007; Seo et al. 2018). The half-life of the most commonly identified PFAS in humans is about 4 to 8 years (Olsen et al. 2007). Contaminated water and food

intake, indoor air inhalation and skin contact with the household environment are likely major exposure routes in human populations (De Silva et al. 2021; Lau 2015; Sunderland et al. 2019). Epidemiological studies have reported that PFAS exposures were associated with various human health risks, including child and adult adiposity and other cardiometabolic functions (Kahn et al. 2020; Rappazzo et al. 2017), cancers (Bartell and Vieira 2021; Steenland and Winquist 2021), fetal growth and childhood neurodevelopmental outcomes (Liew et al. 2018), and immunological health conditions (Chang et al. 2016). However, the underlying biological mechanisms how the exposures lead to these reported adverse health outcomes remain inconclusive.

Toxicological studies in laboratory animals or human cell lines have examined potential mechanisms of action for PFAS exposure, which include elevated oxidative stress (Chen et al. 2017b; Liu et al. 2016), shift from carbohydrate metabolism to fatty acid oxidation (Bjork et al. 2011), nuclear lipid hyperaccumulation (Li et al. 2017), suppression of glutamate-related neurological pathway (Wang et al. 2019), and loss of gap junction intercellular communication (Upham et al. 2009). Some metabolomics studies in *in vivo* or *in vitro* models have associated PFAS exposure with the metabolism of lipids, amino acids and purines (Gong et al. 2019; Ortiz-Villanueva et al. 2018; Zhang et al. 2021), but the dosage in experimental studies might not be comparable to exposure of the general population. Human studies of biological responses associated with PFAS exposures have predominantly focused on selected and targeted lipid or hormone markers or biomarkers of liver function (Donat-Vargas et al. 2019; Geiger et al. 2014; Gleason et al. 2015). In addition, increasing numbers of epidemiological studies have employed non-targeted metabolomics to identify early effect predictors for health risks due to PFAS exposure, but no studies to date have evaluated the methodology and robustness of findings in these environmental metabolomics studies.

The purpose of this scoping review is to provide an assessment of the current evidence regarding non-targeted metabolomics and associations with PFAS exposure in humans. We evaluated the study characteristics, research methods, metabolites identified, and metabolic pathways reported to be associated with specific PFAS chemicals in these studies. We also identified knowledge gaps and made recommendations for future research that examines alterations of the human metabolome associated with PFAS and related environmental chemical exposures.

2. Material and methods

We conducted a scoping review in compliance with the PRISMA methodology for Scoping Reviews (Tricco et al. 2018). This review summarized the literature on epidemiological studies through database searching (PubMed, Dimensions, Web of Science Core Collection as licensed at Yale, Embase via Ovid, Scopus), and citation chaining (via Citationchaser (Haddaway et al. 2021), which uses Lens as its data source). The searched terms used in PubMed were: (PFAS[tw] OR PFASs[tw] OR perfluoro*[tw] OR polyfluoro*[tw] OR PFOS[tw] OR PFOA[tw] OR PFNA[tw] OR PFHxS[tw] OR PFSA[tw] OR PFCA[tw] OR PFOSA[tw] OR PFDA[tw] OR PFUnDA[tw] OR PFDeA[tw] OR PFDoA[tw] OR PFHpA[tw] OR PFUdA[tw] OR EtFOSAA[tw] OR MeFOSAA[tw])

AND (metabolomics[MeSH Terms] OR metabolome[MeSH Terms] OR metabolomics[tw] OR metabolic[tw] OR metabolome[tw] OR metabonomics[tw]) AND (non-targeted[tw] OR non-target[tw] OR untargeted[tw] OR untarget[tw] OR mwas[tw] OR metabolome-wide[tw]) NOT (animal[mh] NOT human[mh]). Similar terms and logic were applied in other databases (Appendix A). We reviewed the titles of all study items found by the search and reviewed abstracts and full articles when necessary to identify studies meeting our inclusion criteria. The inclusion criteria were: 1) published epidemiological original articles, 2) publication date range from the year 2000 to 1st April 2021, 3) no language limit, 4) with at least one type of PFAS measured in participants' biofluid samples, 5) with non-targeted metabolomics applied in participants' biofluid samples.

Information was abstracted based on the publication in print and any further appendices provided by the authors using a tabular format. We extracted study characteristics, including study design, country/location (with project names if applicable), study population, sample size, measured PFAS types, sample collection, analytical platform of both PFAS exposure and metabolome, statistical analysis, and main findings on metabolomic features and pathways. In summarizing study findings related to exposure and metabolomic associations, we focused on the four most studied PFAS compounds which were reported in at least three studies (PFOS (n=11), PFOA (n=10), PFHxS (n=9), and PFNA (n=5)) and well-defined PFAS mixtures (n=7). The recent scientific evaluation from European Food Safety Authority (EFSA) has also focused on these four PFAS because they are more comparable in terms of observed levels in human blood, as well as several toxicokinetic effects and health effects in animals (Chain et al. 2020). We first presented the significant metabolomic features and pathways (along with defined statistical significance levels) reported in each study. Whenever available, we recorded metabolomic features with identification confidence levels in the Metabolomics Standards Initiative (MSI) reporting criteria (Schymanski et al. 2014; Sumner et al. 2007). Briefly, the confidence of identification is often communicated with the following five identification levels: fully identified compounds (level 1); putatively annotated compounds (level 2); putatively characterized compound classes (level 3); unassigned compounds (level 4-5) (Schymanski et al. 2014; Sumner et al. 2007). Level 1 identifications were confirmed via an accurate matching on a reference standard with MS, MS/MS and retention time. Level 2 features were identified based on accurate mass and fragmentation patterns using mass spectra in literature or external laboratory data without retention time information. Level 3 features were assigned to a chemical class rather than the exact structure, using a combination of accurate mass, mass spectra and fragmentation pattern knowledge, and retention time window. Level 4 and 5 features were those unable to be assigned with a possible structure, but only molecular formula or exact mass could be affirmed. In our main table (Table 1), we listed confirmed level 1 features, or all assigned features (level 1-3) when specific confidence levels were unavailable, while unassigned compounds (level 4-5) were excluded in this study. Next, we counted the overlap of reported significant associations between aforementioned four individual PFAS or PFAS mixtures and metabolites/pathways across studies. We used stacked barplots in the *ggplot2* R package to present the findings (R Development Core Team 2021; Wickham H 2016). A significant association included was defined as a reported false discovery rate (FDR) value <.05 if not otherwise specified in Table 1 or its footnotes. All assigned metabolomic features (level 1

to 3) were also included in the quantitative description, and the features/pathways associated with PFAS were summed by the study numbers. We presented the results for individual PFAS or PFAS mixtures separately. Definitions of PFAS mixtures in each applicable study could be found in Supplementary Table 1. For visualization, metabolites and metabolic pathways were grouped based on chemical taxonomy in human metabolome database (HMDB) (https://hmdb.ca/) and metabolism category in Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), and ranked by the total number of studies regardless of associated PFAS types.

We utilized a scoring tool to evaluate the methodology and report quality of included studies, based on existing guidelines (Barnes et al. 2016a; Barnes et al. 2016b; Jin et al. 2021; Spicer et al. 2017; Sud et al. 2016; van der Werf et al. 2007). Five metrics were used including a total score of six (Supplementary Figure 1). The scores for each study were assessed independently by two authors (PG and QY) and subsequently confirmed by a third author (ZL).

3. Results

Figure 1 shows the process of study inclusion and exclusion using the PRISMA 2009 Flow Diagram. We identified a total of 11 eligible publications (2017-2021) written in English. Nine of the included publications were identified through the database searches, and two additional included publications were extracted through citation chaining. Most of the studies reviewed were scored (>3) for having a moderate to high methodological and reporting quality (Supplementary Table 2). Briefly, all studies have provided full description or relevant references on the study populations, the sample collection, and the analytical methods for PFAS exposure measurement. Three studies did not report complete information for data processing workflow including imputation of missing values (Alderete et al. 2019; Hu et al. 2019; Jin et al. 2020), but software used (*e.g.*, xMSanalyzer) was reported. Five studies (Alderete et al. 2019; Jin et al. 2020; Li et al. 2020; Lu et al. 2019; Wang et al. 2017a) did not clearly state certainty of metabolite identification or did not capture high confidence metabolites (level 1-2).

3.1 Study designs and populations

Table 1 presents the study characteristics of the included studies. Study samples were collected from nested case-control studies (n=3), prospective cohorts (n=4), cross-sectional studies among occupational workers (n=2) or in high-risk group (*i.e.*, obesity history without diabetes, n=1) or patients of a specific disease (*i.e.*, non-alcoholic fatty liver disease, n=1). The number of study participants in these studies ranged from 40 to 965. The age groups covered were broad and included children and adolescents (Alderete et al. 2019; Jin et al. 2020; Kingsley et al. 2019), young adults (Chen et al. 2020), middle-aged adults (Lu et al. 2019; Schillemans et al. 2020; Wang et al. 2017a), and elderly (Salihovic et al. 2019). There were three studies focused on pregnant women (Hu et al. 2019; Li et al. 2020; Maitre et al. 2018). The eleven studies were conducted in the United States (n=6), in Europe (n=3), and in China (n=2).

Nine studies assessed cross-sectional PFAS and metabolome associations using samples collected at the same study date. One study evaluated baseline PFAS exposure and metabolite changes over an average of 1.3 years of follow-up (Alderete et al. 2019). A pregnancy study assessed PFAS at baseline and metabolomic profiles twice in the first and the third trimesters (Maitre et al. 2018). Details of statistical models and covariate information are presented in Supplementary Table 3.

Four studies were designed to investigate metabolites or metabolic markers associated with PFAS exposures and also their relationship with metabolic health outcomes. Findings of these four studies are presented in Table 2. The investigated health outcomes included non-alcoholic fatty liver disease (NAFLD) (Jin et al. 2020), alterations in glucose homeostasis (Alderete et al. 2019), type 2 diabetes (T2D) (Schillemans et al. 2020), and cardiometabolic outcomes such as oral glucose tolerance test (OGTT) measures, body fat and lipid profiles (Chen et al. 2020).

3.2 PFAS exposure assessment

Table 1 shows that 2 to 11 types of PFAS were investigated in these 11 studies. The most studied PFAS compounds (in at least 3 studies) were PFOS (n=11), PFOA (n=10), PFHxS (n=9), and PFNA (n=5). PFAS levels were measured in human plasma (n=6) or serum (n=5) samples. Among three studies in pregnant women, one study measured PFAS in the first-trimester serum samples (Maitre et al. 2018) while two studies used serum samples throughout pregnancy and/or from early postpartum period (1-3 days after delivery) (Hu et al. 2019; Li et al. 2020). In addition to studying individual PFAS compounds, seven studies evaluated PFAS mixtures (Supplementary Table 1). Three of these studies computed principal components as a composite variable representing PFAS burden (Alderete et al. 2019; Jin et al. 2020; Schillemans et al. 2020), two studies calculated “total PFAS” by a molarity sum of all the detected PFAS (Lu et al. 2019; Wang et al. 2017a), and one study constructed total PFAS using multivariable multiple linear regression (MMLR) (Salihovic et al. 2019).

All studies employed MS coupled with liquid chromatography (LC) to measure PFAS compounds (Table 1). Additional analytical details regarding PFAS exposure assessments can be found in Supplementary Table 4. All studies reported the overall frequencies of detection for each PFAS and only two studies did not report the limits of detection (Hu et al. 2019; Maitre et al. 2018). Eight studies have provided references for quality assurance and quality control procedures. In general, PFOS was detected at the highest concentrations in most studies, followed by PFOA. The highest concentrations of PFOS appeared in the China-based occupational cohort in 2017 (median ~909 ng/mL) (Wang et al. 2017a), followed by pregnant women in the U.S. CHDS cohort with samples collected in the 1960s (median ~42 ng/mL) (Hu et al. 2019; Li et al. 2020; Wang et al. 2011), and in the middle-aged adults from North Sweden in 1990s (median ~20 ng/mL) (Schillemans et al. 2020). The lowest concentrations of PFOS (median ~4 ng/mL) were reported in a study of U.S. children and adolescents between 2007 and 2015 (Jin et al. 2020). These findings are consistent with the temporal changes in perfluorinated compounds reported previously (Kato et al. 2011; Liu et al. 2021; Nyberg et al. 2018).

3.3 Non-targeted metabolomics analysis

Among all eleven studies, ten studies conducted non-targeted metabolomics analysis in plasma (n=6) or serum (n=4). Only one study (in pregnancy) examined urinary metabolomic profiles (Maitre et al. 2018).

For analytical platforms, ten studies used LC-MS for metabolomics in plasma or sera and one of them additionally used gas chromatography–mass spectrometry (GC-MS) (Lu et al. 2019). One urinary metabolomics (Maitre et al. 2018) analysis was conducted using ^{1}H NMR spectroscopy (Beckonert et al. 2007). LC-MS appears to be the most common approach for metabolomic analyses related to PFAS exposures (Soltow et al. 2013), but there were various characteristics regarding types of ionization (*i.e.*, electrospray ionization, electron ionization), modes of ionization (*i.e.*, positive, negative), mass analyzer (*i.e.*, orbitrap, quadrupole time-of-flight (QToF)), and types of columns. For mass analyzer, an orbitrap was used in eight studies and two used the QToF. Following LC–MS, six studies extracted raw data files and aligned them using apLCMS (Yu et al. 2009; Yu et al. 2013) with modifications by xMSanalyzer R package to improve feature detection, quality assessment, and annotation (Uppal et al. 2013). Three studies (Lu et al. 2019; Salihovic et al. 2019; Schillemans et al. 2020) took raw data into XCMS data processing workflow implemented in R (Ganna et al. 2015; Shi et al. 2017). One (Wang et al. 2017a) used SIEVE software to process the raw data (Zhang et al. 2014).

All studies have described the quality control and quality assurance approaches for the metabolomics analysis undertaken. Four studies reported that all individual samples were analyzed in triplicate (Chen et al. 2020; Hu et al. 2019; Jin et al. 2020; Kingsley et al. 2019), while this information was unclear in the other studies included. Only one study (Hu et al. 2019) included internal standards for non-targeted metabolomics analysis. Among the studies reviewed, three studies (Alderete et al. 2019; Hu et al. 2019; Jin et al. 2020) did not report the methods used for missing value data imputation, data normalization, or transformation. Different tools were used to address batch effects, including a cluster-based approach that calculated measurement drift per batch with *batchCorr* R package (Brunius et al. 2016), or using the ComBat method in *xMSanalyzer* software to correct the m/z features by batches (Johnson et al. 2007; Uppal et al. 2013). One study (Schillemans et al. 2020) used the former tool, three studies (Alderete et al. 2019; Jin et al. 2020; Kingsley et al. 2019) applied the latter method. It is unclear whether the three other studies that used *xMSanalyzer* software (Chen et al. 2020; Hu et al. 2019; Li et al. 2020) also conducted the ComBat method as this was not reported. Details regarding batch correction were not summarized in the remaining studies (Lu et al. 2019; Salihovic et al. 2019; Schillemans et al. 2020; Wang et al. 2017a). The heterogeneity in reporting for metabolomics studies has been previously noted, and suggestions have been made for improving reporting practices (Peter et al. 2021; Rattray et al. 2018).

3.4 Metabolite alterations associated with PFAS exposure

Eight studies reported significant associations between four dominant types of PFAS or PFAS mixtures and metabolites (identified to MSI levels 1-3), while the other three studies did not show feature-level associations (Alderete et al. 2019; Kingsley et al. 2019; Li et

al. 2020). The full list of metabolites associated with PFAS is presented in Supplementary Table 5. For metabolite compound classes reported to be associated with PFAS exposures are in at least three studies, the numbers of associations organized by the compound class, the category of metabolite within the class, and the types of PFAS are shown in Figure 2.

A total of 6 studies reported associations between PFAS and amino acid-related metabolites (Figure 2). Two specific amino acid-related metabolites were suggested in multiple studies which included pyroglutamic acid (Lu et al. 2019; Wang et al. 2017a) and either glutamate/glutamine (Chen et al. 2020; Hu et al. 2019), but the reported associations varied in both positive or negative directions (Supplementary Table 5). In addition, PFAS were also associated with lipids and lipid-like metabolites in the fatty acid esters or fatty acids and related conjugate classes. The specific lipid-related metabolites most commonly reported are carnitines/acylcarnitines (Chen et al. 2020; Hu et al. 2019; Lu et al. 2019) and glycerophosphocholines (Chen et al. 2020; Lu et al. 2019; Salihovic et al. 2019) . Most of these associations reported were related to PFOS and PFOA exposures while a few metabolites were detected for PFHxS or the mixture of PFAS compounds.

3.5 Metabolic pathway alterations associated with PFAS exposure

Nine studies conducted pathway analysis to identify key altered metabolic pathways associated with PFAS exposure. Six of them used *Mummichog* pathway enrichment analysis (Li et al. 2013), which can directly map metabolites predicted in high-throughput metabolomics data to known metabolic networks and predicts functional activity (Li et al. 2013). One study (Salihovic et al. 2019) performed pathway enrichment and topology analysis using MetaboAnalyst 3.0 (Xia and Wishart 2016). One study (Lu et al. 2019) conducted pathway analysis using the KEGG database (http://www.genome.jp/kegg/). While one study (Wang et al. 2017a) did not provide information on the tools used for pathway analysis.

The full list of altered metabolic pathways significantly associated with four dominant PFAS subtypes or mixtures are shown in Supplementary Table 6. For metabolic pathways reported to be associated with PFAS in at least three studies, the numbers of associations organized by the category of metabolism, specific metabolism pathways, and the types of PFAS are shown in Figure 3.

Similarly, amino acid metabolism and lipid metabolism were the most commonly suggested metabolic pathways associated with PFAS exposure (Figure 3). For amino acid metabolism, urea cycle/amino group metabolism (Alderete et al. 2019; Hu et al. 2019; Jin et al. 2020; Li et al. 2020; Lu et al. 2019), tryptophan metabolism (Chen et al. 2020; Kingsley et al. 2019; Li et al. 2020; Lu et al. 2019), valine, leucine and isoleucine degradation (Hu et al. 2019; Jin et al. 2020; Kingsley et al. 2019; Li et al. 2020), glycine, serine, alanine and threonine metabolism (Alderete et al. 2019; Hu et al. 2019; Jin et al. 2020; Kingsley et al. 2019), and beta-alanine metabolism (Alderete et al. 2019; Hu et al. 2019; Kingsley et al. 2019; Li et al. 2020) were associated with multiple PFAS exposure types and for PFOS was reported in at least two studies. For lipid metabolism, glycerophospholipid metabolism (Chen et al. 2020; Jin et al. 2020; Kingsley et al. 2019; Li et al. 2020; Salihovic et al. 2019), glycosphingolipid metabolism (Alderete et al. 2019; Chen et al. 2020; Kingsley et

al. 2019; Li et al. 2020) , de novo fatty acid biosynthesis (Alderete et al. 2019; Chen et al. 2020; Kingsley et al. 2019), fatty acid activation (Chen et al. 2020; Kingsley et al. 2019; Li et al. 2020), they were associated with various PFAS exposure types and for both PFOS and PFOA were reported in at least two studies. Additional pathways were also suggested, such as carbohydrate metabolism (*e.g.*, TCA cycle), nucleotide metabolism (*i.e.*, purine and pyrimidine metabolism), and other pathways relevant to vitamins and xenobiotics metabolism. These metabolic pathways were linked with each of the individual PFAS compounds and their mixtures. These metabolic pathways were not specific to either plasma or serum samples (Supplementary Figure 2). Although there were only three studies in children and adolescents, this group covered metabolic pathways that were also reported for adults and pregnant women. The *Mummichog* algorithm contributed the most to the metabolic pathway summary, which also overlaps with pathways reported from using other statistical algorithms.

4. Discussion

The 11 studies we reviewed demonstrated the increasing utilization of the non-targeted metabolomics approach to screen for alterations of the human metabolome associated with specific and mixtures of PFAS exposure in wide demographic groups. We identified some overlapping PFAS exposure and metabolomic associations reported across studies, but these results were synthesized from a relatively low number of eligible studies thus require further scrutiny. Future studies are needed to investigate potential differences across demographic subgroups and research methodology and explore whether the biological pathways altered by PFAS exposures could lead to specific clinical outcomes using longitudinal designs. Standardization of research approach and reporting of metabolomic results (*e.g.*, data processing workflow, confidence levels of metabolite identification, and pathway analyses procedures) are important to support cross-study comparisons. Potential influences from systematic errors, such as confounding bias and measurement errors, also need attention in future research.

Previous epidemiological research have suggested the effects of multiple types of PFAS on altering amino acid and lipid metabolism and cardiometabolic health risks (Chen et al. 2017a; Cheng et al. 2012; Chou et al. 2018; Farthing et al. 2015; Kovalik et al. 2021; Martinez et al. 2020; Qi et al. 2017; Senyavina et al. 2013). In non-targeted metabolomic associations we evaluated, a top-hit pathway related to PFAS exposure in the reviewed studies was urea cycle/amino group metabolism, which is an indispensable pathway to dispose of the excess nitrogen in the body (Alemany 2012; Ramos-Tovar and Muriel 2017). The disruption of urea cycle metabolism has implications on insulin resistance (Cao et al. 2019; Li et al. 2010) and diabetes-related heart failure in older adults (Kovalik et al. 2021; Razavi et al. 2020; Urpi-Sarda et al. 2019). Among metabolites involved in lipid metabolism associated with PFAS, carnitines/acylcarnitines are major molecules in the energy supply pathway of long chain fatty acid β-oxidation. Impaired fatty acid oxidation along with tissue lipid accumulation has been recognized as critical in the pathophysiology of obesity and insulin resistance (Bene et al. 2018; Mihalik et al. 2010), and cardiomyopathy (Kompare and Rizzo 2008; Wang et al. 2018). Another top-hit lipid pathway associated with PFAS exposure was glycerophospholipid metabolism, which has been previously linked to

CVD progression (Chen et al. 2021), T2D and tuberculosis comorbidity (Lopez-Hernandez et al. 2019). Overall, these findings which associate PFAS exposure with changes to the endogenous metabolome corroborate results from epidemiological studies, suggesting that PFAS exposures might affect cardiometabolic health outcomes across life-span from childhood (Li et al. 2021a; Manzano-Salgado et al. 2017) to adulthood (Valvi et al. 2021; Zeeshan et al. 2021).

The metabolomic findings also implied that PFAS could have biological effects on several other human organs and physiological processes. For the kidney, one of the most significantly changed pathways for amino acid metabolism in the reviewed studies - tryptophan metabolism, is a sensitive and reliable indicator of renal injury, as the amount of L-tryptophan decreases with the development of chronic kidney disease (Chou et al. 2018; Gong et al. 2019). Abnormalities of purine and pyrimidine metabolism could also lead to hyperuricemia (Gong et al. 2019; Lv et al. 2013; Rathmann et al. 1998), a risk factor of chronic kidney disease (Gong et al. 2019). A growing body of toxicological and epidemiological evidence has indicated that PFAS compounds are emerging environmental threats to kidney health (Stanifer et al. 2018). In terms of the liver, some aberrant amino acids (*e.g.*, glutamate/glutamine) and amino acid derivatives (*e.g.*, pyroglutamic acid) have been recognized as prominent factors for the development of nonalcoholic steatohepatitis (Qi et al. 2017). Impaired fatty acid metabolic pathways included muscle damage (Lehmann et al. 2010) and hepatic dysfunction (Kompare and Rizzo 2008). The liver toxicity of PFAS has been widely documented in animal literature and recently also in clinical studies (Cave 2020; Gleason et al. 2015; Wu et al. 2018). Concerning the central nervous system, glutamate is a major excitatory neurotransmitter in the central nervous system which is critical for neuronal growth and maturation (Wang et al. 2019). The glycerophospholipid metabolism could affect fetal growth (Morillon et al. 2021), the brain neural membrane functions (Farooqui et al. 2000), and neuropsychiatric diseases (Healy-Stoffel and Levant 2018; Kalkman et al. 2021; Lin et al. 2010; Young and Conquer 2005). Finally, the TCA cycle and nucleotide metabolism (*i.e.*, purine and pyrimidine metabolism) are critical for cellular homeostasis and energy generation related mechanisms (Martinez-Reyes and Chandel 2020; Nyhan 2005). A defective TCA cycle has been linked with a variety of clinical diseases in mechanistic studies, ranging from encephalopathies, neurodegenerative diseases, to cancers (Briere et al. 2006).

4.1 Quality of evidence and risk of bias

Approximately 40% of the metabolic pathways associated with PFAS exposure identified in this review were reported in at least three studies, and other sporadic and inconsistent reports could be affected by methodological issues, chance errors, or population differences. Although most studies reviewed received moderate to high methodological and reporting quality, an unstandardized data processing workflow for non-targeted metabolomics data, and the uncertainty of metabolite identifications bring challenges for findings synthesis across studies. First, the low quality of sample collected may introduce measurement errors, and information provided from each study generally did not allow the direct assessments of the influence of sample collection and storage. We may not rule out measurement errors of exposures for each individual study, which is a common issue for any meta-analyses. Quality

control procedures should be described in all studies. Strengths and limitations of the two predominantly utilized analytical platforms for data acquisition and processing (i.e., MS and NMR spectroscopy) have been summarized elsewhere (Aderemi et al. 2021). Briefly, NMR spectroscopy has advantages in the reproducibility of result, which is also more quantitative, in addition the technique is non-destructive to the sample and is easier to prepare, whereas the MS approach provides much wider metabolite coverage and that comprehensive metabolite databases have been developed for metabolite identification. The raw data output from both MS and NMR spectroscopy requires *pre-processing*, including peak selection and alignment, baseline correction, and *data preparations* such as normalizing, centering, removing outliers and transforming data (Hivert et al. 2015). In addition, missing value imputation and correction for batch effects are also crucial to consider. Standardizing these data steps, and reporting, is important for cross-study comparison and synthesis.

Another important consideration is *metabolite identification*. Recent advances for metabolite identification have been summarized elsewhere (Nguyen et al. 2019a). To date, the most common method utilized for non-targeted studies is the traditional ion-centric approach, where the signals are compared to a standard reference library, such as the HMDB or METLIN (Montenegro-Burke et al. 2020) to identify metabolites (Hivert et al. 2015). However, given the large number of metabolites and the broad range of chemistries in reality, no reference library is complete to date (Nguyen et al. 2019a). Without stable isotope-labeled internal standards added to each sample for specific chemicals of interest, the non-targeted metabolomics analysis could have larger measurement errors in quantification but non-targeted approach has the advantage of being more feasible and cost-efficient compared to the targeted metabolomics approach (Chen et al. 2020). This is a general concern for current metabolomics studies in all fields which requires further development of metabolite identification techniques and the standardization of reporting.

In comparison, the *exposure assessment* methods and reporting of the common PFAS compounds in the reviewed studies were more consistent. All studies measured PFAS in blood plasma or serum, which has a 1:1 plasma to serum ratio in humans (Carpenter David et al. 2002; Ehresman et al. 2007). The studies did not utilize other methods to measure PFAS levels that might generate more measurement errors, such as geospatial modeling (Guelfo et al. 2018). The four main compounds of interest in this review generally had very high detection rates, but data steps to handle the levels below the limit of detection varied. A major issue in PFAS exposure assessment is the heterogeneity in the definition of PFAS mixture. Strategies to study environmental mixtures have evolved over time and the statistical modeling techniques are expected to advance (Carpenter David et al. 2002; Weisskopf et al. 2018). Only two of the eleven studies (Alderete et al. 2019; Maitre et al. 2018) collected repeated biological samples with a short time gap between the PFAS assessment and the measures of the metabolome.

Since metabolomics studies can identify thousands of features, multiple testing in statistical analyses needs to be considered. Findings for single/sporadic metabolites are subject to false positive results (type I errors) without multiple testing correction, but false negative results (type II errors) could occur when a strict statistical significance adjustment threshold was used. Pathway enrichment approaches that focus on *multiple hits* on the same metabolic

pathways were suggested to be more robust against false positive findings and yield more consistent and replicable findings (Khatri et al. 2012; Nguyen et al. 2019b). This also underscores a key point to establishing the validity of pathway results –accurate identification of the upstream metabolites, as discussed above. Different methods for pathway enrichment analysis used could create discrepancies for cross-study comparisons. In our review, seven studies conducted pathway enrichment analysis based on an established algorithm within the software (*e.g.*, the *Mummichog* algorithm), thereby the common findings are mostly driven by a single pathway analysis algorithm. The *Mummichog* program uses databases which encompass thousands of human metabolites such as KEGG, Recon1, and the Edinburgh human metabolic network, therefore the assignment of metabolites to pathways is based on canonical knowledge of metabolite pathways. Some pathways reported to be associated with PFAS exposure could also be affected by other environmental contaminants (Heindel et al. 2016; Li et al. 2021b), as many of the detoxification pathways are shared, and many of the contaminants are sex-steroid hormone receptor disruptors that share similar effects. Some might argue that this algorithm may systematically lead to ubiquitous endogenous pathways closely linked to oxidative stress and systematic inflammation (Li et al. 2021b; Samet and Wages 2018). However, three studies that did not use *Mummichog* algorithm also reported amino acid and lipid metabolism associated with PFAS exposure (Lu et al. 2019; Salihovic et al. 2019; Wang et al. 2017a), and these metabolic pathways were also detected in the studies using *Mummichog* algorithm (Supplementary Figure 2). Our exploratory approach is a timely initial assessment to look for overlaps in metabolomics signatures associated with PFAS exposure based on available evidence. Future mechanistic studies are needed to confirm these findings.

Systematic errors, particularly confounding bias, could influence the findings we reviewed. There is a general confusion in the epidemiological literature on whether the research performed is designed to conduct prediction modeling or to estimate the (causal) effect from defined exposures. If the study objected is to estimate exposure effect, confounding adjustment could follow rules and methods outlined in causal modeling, guided by knowledge in the literature (i.e., use of the directed acyclic graphs to select covariates which was only performed in one of the studies reviewed (Alderete et al. 2019). Generally, diet, lifestyle factors, and the underlying health status of the participants are key potential confounders when estimating PFAS exposure effects on health (Eick et al. 2021; Seshasayee et al. 2021). Demographic-specific variables, such as occupational factors or reproductive history in pregnancy cohorts, should be considered depending on the study setting. Confounding by other environmental pollutants can potentially influence the reported findings (Samet and Wages 2018), but a blind adjustment for multiple highly correlated exposure variables is not encouraged because that would lead to other problems such as a decrease in statistical efficiency and even the risk for bias amplification (Weisskopf et al. 2018). Other sources of bias should also be considered, including survival bias if PFAS exposures can influence mortality in the study setting (Liew et al. 2015; Mastrantonio et al. 2018), and measurements errors that influence both the exposure and the outcome variables. The literature in this field thus needs to pay more attention to these potential problems when correlating PFAS exposure data with markers generated from the human metabolome in order to derive valid inference.

4.2 Limitations of this review

This present review should be considered with several limitations. First, our review was limited by the number of eligible studies, heterogeneity in definitions of PFAS mixtures, and a wide confidence in the robustness of metabolite identification (using MSI levels 1 to 3) detected from the individual studies. In the description of study characteristics, there were some limits on the level of details provided by the individual reports. Moreover, our findings for the PFAS-associated metabolite and metabolic pathway changes across studies would be useful to point towards future investigation, but they should not be used to draw a definite conclusion with the current evidence available. As discussed above, there was considerable heterogeneity in the research methods in the current literature, including the definition for PFAS mixture, the data processing steps and identification of metabolites, and the approaches used for pathway enrichment analyses. Our approach prioritizes on summing and ranking the common and overlapping metabolite features or pathways associated with specific PFAS compounds from the articles we reviewed. This approach, however, would not capture true exposure-metabolomic associations that are demographic and health outcomes specific, *e.g.*, true specific exposure-outcome associations that are not expected to show up across demographic groups from a wide range of studies.

4.3 Recommendations for future research

The findings of our review have identified a few research gaps and highlighted several important areas for future studies. First, longitudinal or repeated measurement of metabolic profiling are needed to distinguish timing-specific or cumulative exposure effects of PFAS on alterations of metabolome. Also, several steps could be taken to further strengthen the confidence and validity of findings derived using metabolomic data. Second, methods that improve the identifications of detected metabolites and the additional use of pathway analysis and replications of findings could rule out false positive results (Cai et al. 2020; Nguyen et al. 2019b). For example, to further investigate PFAS-related exposure-disease mechanisms, a “meet-in-the-middle” approach could be explored, *e.g.*, studying PFAS, metabolome, and disease phenotypes relationships in the same study population (Chadeau-Hyam et al. 2011; Jin et al. 2020; Schillemans et al. 2020). Also, an incorporation of causal mediation analyses using metabolomic data might help to statistically quantify the biological mediating pathways between the association of an environmental exposure and the health outcome (Inoue et al. 2020). Third, biases that can threaten the validity of findings should also be addressed or acknowledged. For instance, all studies reviewed were in observational nature, but few considered potential biases due to confounding, selection bias and measurement errors. An incorporation of statistical methods that could be used to evaluate or adjust for these biases when analyzing high-dimensional biologic data in human cohorts are needed (Misra et al. 2019). More potential confounding factors should be evaluated in future studies, such as factors that can determine the level of PFAS exposures (*e.g.*, diet, occupation, socio-economic profile, co-exposures to other chemicals, and chronic health conditions of participants) and influence the metabolomic signals. Studies of co-pollutant exposures are needed, but blindly co-adjusting multiple correlated exposure variables in one regression model should be avoided (Weisskopf et al. 2018). Forth, it is difficult to standardize the non-targeted metabolomic workflow between research labs due to the various analytical platforms and bioinformatics approaches used, and the inherent

complexity of the metabolome for which there is not a one-size-fits-all approach to identify all metabolites within a sample. However, some standardization within the workflow would add robustness, improve the study quality of metabolomics research, and facilitate comparisons of findings across studies (Hernandez-Mesa et al. 2021). Fifth, research of the newer or less studied PFAS compounds with clear reports on quantification procedures (*e.g.*, analytical platforms, LOD, frequencies of detection, missing data imputation) are needed. The studies we reviewed focused on the most common types of PFAS and their mixtures, while recent reports have suggested there are thousands of fluorinated chemicals have been manufactured and used (OECD 2018). The real PFAS body burden is underestimated when not counting for the emerging replacement of other major types of PFAS. Currently, no human studies could accurately quantify all possible PFAS chemicals, and this is awaiting advancement. Finally, literature review of metabolomics studies may benefit from using citation chaining tools and searching synonyms of "non-targeted metabolomics" thoroughly. For instance, the application of non-targeted metabolomics was described as "hypothesis-generating" or indicated by the analytical platform "orbitrap" in some studies (Lu et al. 2019; Wang et al. 2017a), which could be missed by using narrowly defined search terms.

5. Conclusions

In summary, high-resolution non-targeted metabolomics are increasingly being used to evaluate the biological impacts of PFAS exposures in epidemiological studies. Our review found that lipid- and amino acid-related pathways were most reported to be associated with four types of PFAS exposures, and that these PFAS-related metabolome alterations could have implications for cardiometabolic health and other chronic disease health risk. However, the overall body of literature is small, and heterogeneity of research methodology related to metabolomics feature identifications, data processing, and statistical output could influence the findings. The use of longitudinal measures, improvement in certainty of metabolite identification, adjustments for confounding and other sources of biases, and a more standardized procedure in reporting metabolomic findings are some key components identified for improvement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α-CEHC
alpha-carboxyethyl hydroxychromanol

γ-CEHC

gamma-carboxyethyl hydroxychroman

BH4
Tetrahydrobiopterin

C18:2-CN
acylcarnitine 18:2

C18:1-CN
acylcarnitine 18:1

DAHA
deoxyarabinohexonic acid

FDR
false discovery rate

GPC
glycerophosphocholine

HILIC
hydrophilic interaction liquid chromatography

HMDB
Human Metabolome Database

HPLC
high performance liquid chromatography

HRMS
high-resolution mass spectrometry

LysoPC
lysoophosphatidylcholine

NFALD
non-alcoholic fatty liver

NMR
nuclear magnetic resonance

NO
nitric oxide

NR
not reported

OGTT
oral glucose tolerance test

OH/DC
hydroxyl-/dicarboxyl- acylcarnitine

POPs
persistent organic pollutants

6:2 Cl-PFESA
6:2 chlorinated polyfluorinated ether sulfonate

EtFOSAA
2-(N-Ethyl-perfluorooctane sulfonamido) acetic acid

MeFOSAA
2-(N-Methyl-perfluorooctane sulfonamido) acetic acid

PFAS
per- and polyfluoroalkyl substances

PFBA
perfluorobutanoic acid

PFBS
perfluorobutanesulfonic acid

PFHxS
perfluorohexane sulfonic acid

PFOS
perfluorooctane sulfonate

PFOA
perfluorooctanoic acid

PFDA
perfluorodecanoic acid

PFNA
perfluorononanoic acid

PFDoA
perfluorododecanoic acid

PFHpA
perfluoroheptanoic acid

PFOSA
perfluorooctanesulfonamide

PFUdA
perfluoroundecanoic acid

QToF
quadrupole time-of-flight

RPLC
reversed phase liquid chromatography

Short-chain non-OH/DC
Short-chain non-hydroxyl-/dicarboxyl acylcarnitine

SPE
solid-phase extraction

TCA cycle
Tricarboxylic acid cycle

T2D
type 2 diabetes

UPLC
ultra-pressure liquid chromatography

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Highlights

- Comprehensive review of research studies on metabolomics of PFAS exposures in humans
- PFAS exposures are associated with disruption of amino acid, energy and lipid metabolism
- Metabolic alterations associated with PFAS are implicated in cardiometabolic health
- Research needs include prospective design, standardized reporting, and risk of bias

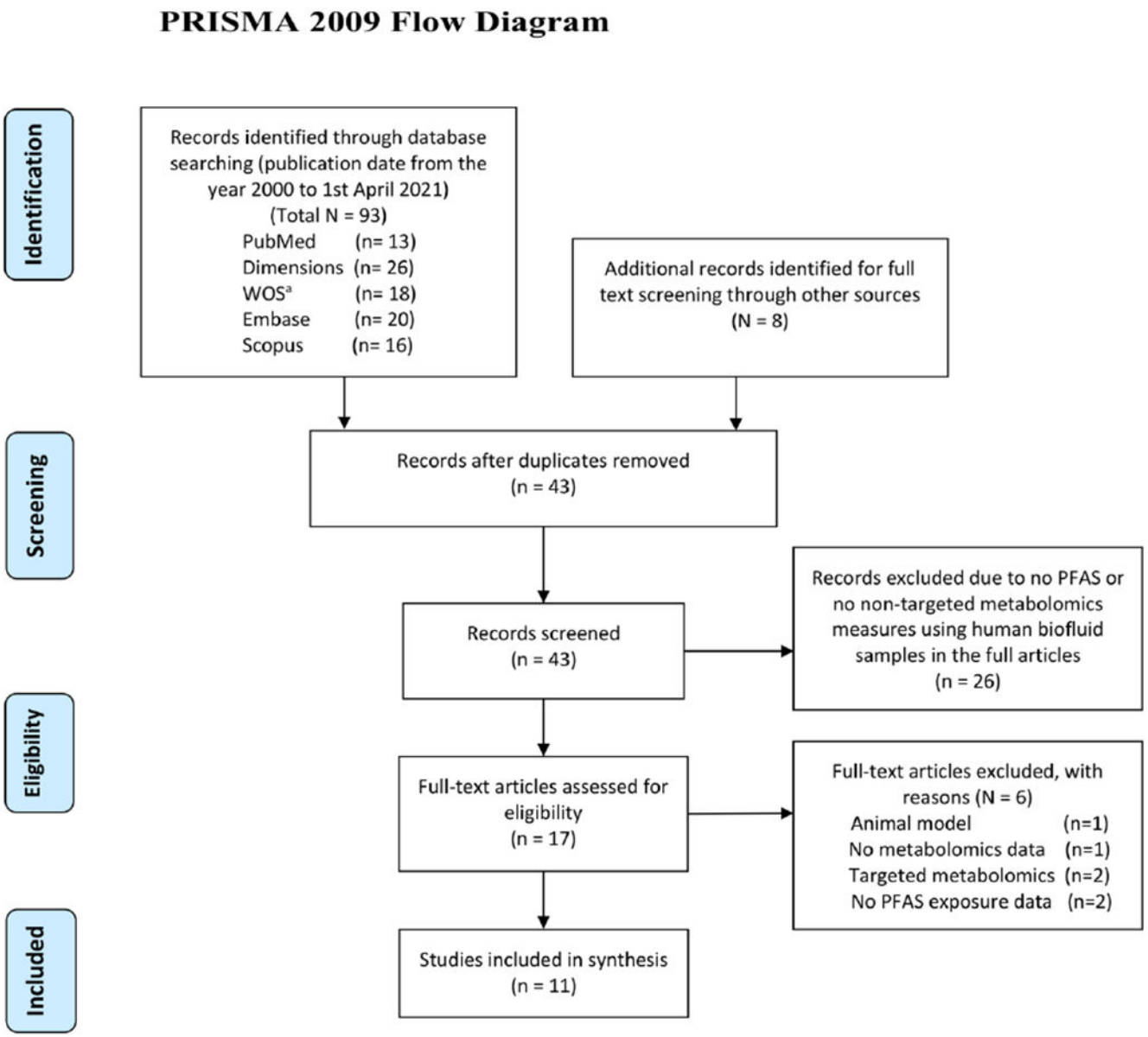

Figure 1. PRISMA Flow diagram depicting process of article selection.

[a] WOS: Web of Science Core Collection as licensed at Yale Institution. Abbreviation: PFAS, per- and polyfluoroalkyl substances.

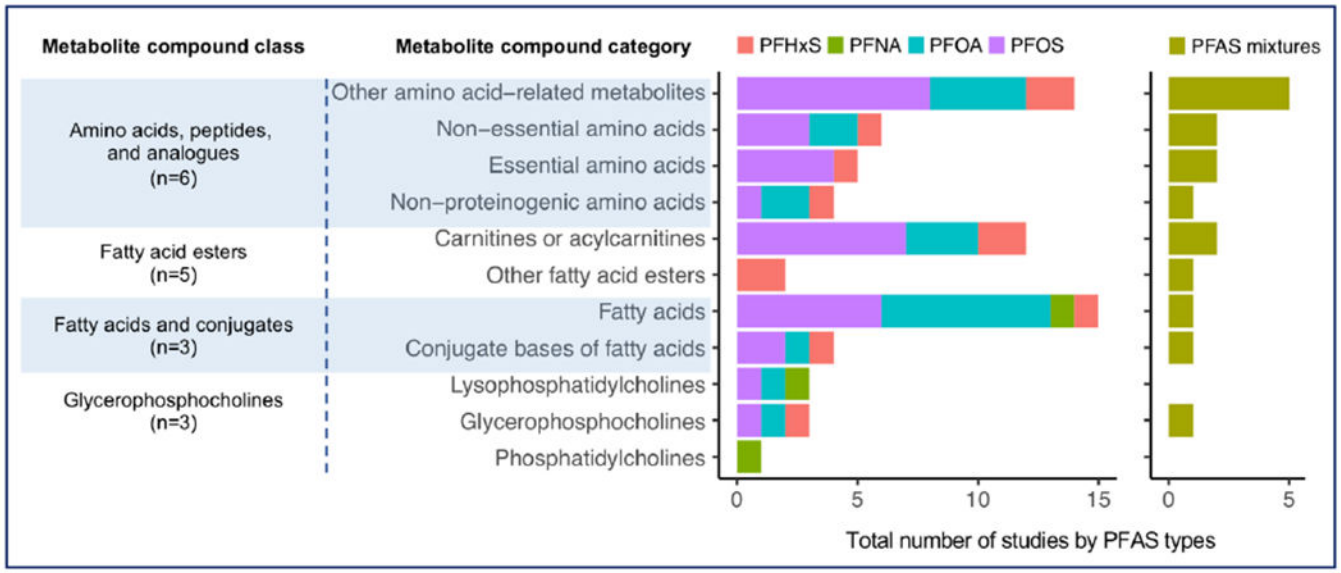

Figure 2. A summary of the commonly detected metabolite compound classes (in at least three studies) and categories associated with four individual PFAS compounds and PFAS mixtures.

Individual PFAS compounds and the mixtures were differentiated in color, and the total number of significant associations summing across PFAS types reported in the eleven studies were organized according to the metabolite compound class and categories in human metabolome database (HMDB). Abbreviations: PFAS, per- and polyfluoroalkyl substances; PFHxS, perfluorohexane sulfonic acid; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid.

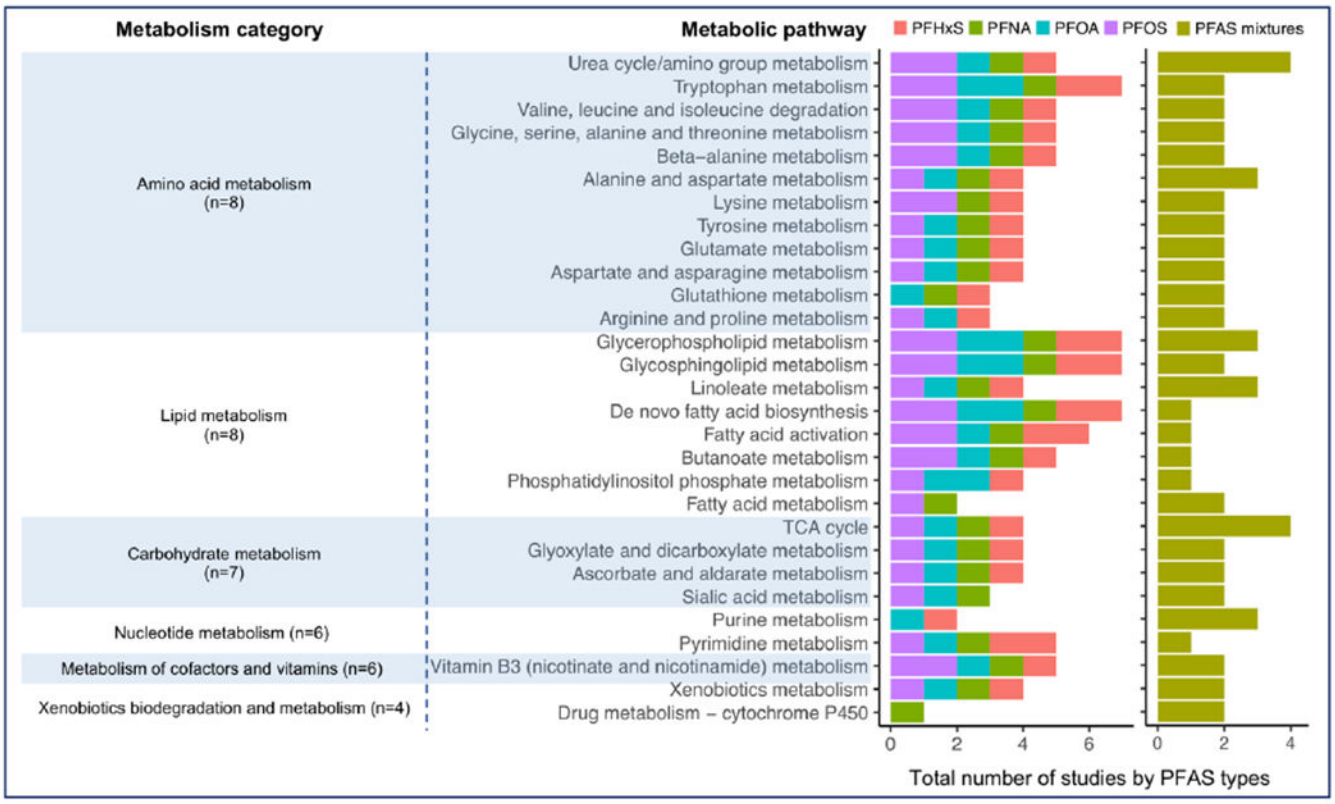

Figure 3. A summary of the commonly detected metabolic pathways (in at least three studies) associated with four individual PFAS compounds and PFAS mixtures.

Individual PFAS compounds and the mixtures were differentiated in color and the total number of significant associations summing across PFAS types reported in the eleven studies were organized according to the metabolism category in Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Abbreviations: PFAS, per- and polyfluoroalkyl substances; PFHxS, perfluorohexane sulfonic acid; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid.

Table 1.

A summary of research methods and major findings reported in the 11 PFAS and non-targeted metabolomic studies.

Publication	Location	Study design and sample size	PFAS and non-targeted metabolome measures[a]	Analytical platform for PFAS and metabolome	Significant metabolomic features associated with PFAS (FDR <0.05[b], confidence levels 1 to 3)	Significant pathways (p for pathway enrichment test <0.05)
(Schillemans et al. 2020)	Northern Sweden	124 cases of type 2 diabetes (T2D) and 124 controls (40-60 years) enrolled during 1990-2003 and followed at 2000-2013	PFHxS, PFOS, PFOA, PFNA, PFDA, PFUdA and metabolome were measured in fasting plasma samples at baseline and follow-up.	PFAS: LC-MS/MS; Metabolome: LC-QToF-MS (Agilent Technologies, USA)	PFAS levels were correlated with 171 features ($0.16 \leq |r| \leq 0.37$). Thirty-five features related to both PFAS and T2D and they predominantly represented glycerophospholipids and diacylglycerols.	n/a
(Chen et al. 2020)	California, US	102 non-diabetic young adults (17-22 years) with a history of overweight/ obese enrolled during 2014-17	PFOA, PFOS, PFHxS and metabolome were measured in fasting and 30-min glucose challenge plasma samples.	PFAS: LC-HRMS Metabolome: Orbitrap LC-HRMS (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific)	Features of confidence level 1: proline, glutamine, methionine, citrulline, mannose, lysoPC (18:0), sphingosine, lactate, oxovalerate, glucose, multiple fatty acids. A targeted metabolomics analysis verified that higher PFOA exposure was associated with higher levels of glycerol (p=0.006) and higher fasting levels of short chain non-OH/DC acylcarnitines (p=0.046).	PFAS were associated with 16 pathways, especially the lipid pathways (*e.g.*, fatty acid activation, glycosphingolipid metabolism) and the amino acid-related pathways (*e.g.*, tryptophan metabolism, arginine and proline metabolism).
(Jin et al. 2020)	Atlanta, Georgia, US	74 youths (7-19 years) with non-alcoholic fatty liver disease enrolled during 2007-15	PFOA, PFOS, PFHxS and metabolome were measured in plasma samples.	PFAS: LC-HRMS Metabolome: Orbitrap LC–HRMS (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific)	Six features (confidence levels 1 to 3), including phosphoethanolamine, tyrosine, phenylalanine, aspartate, creatine, betaine.	PFAS were associated with 21 pathways. The most affected pathways included tyrosine metabolism, aspartate and asparagine metabolism, glycine, serine, alanine and threonine metabolism.
(Li et al. 2020)	California, US	404 pregnant women (an average age 25 years) enrolled during 1959-67. Fifty of these women developed breast cancer.	PFOS, PFOA, PFHxS and metabolome were measured in serum.	PFAS: online SPE-HPLC-MS/MS; Metabolome: Orbitrap LC–HRMS (Thermo Q-Exactive (Thermo Fisher, San Diego, CA))	[c] Metabolite communities identified by a hierarchical community network model associated with the exposures were non-specific and shared among exposures. The most significant connections are shared with the lipid community.	[b] Metabolite communities associated with PFAS community (PFAS mixture) were enriched for fatty acid and mitochondria related pathways.
(Kingsley et al. 2019)	Ohio and Kentucky, US	115 liveborn singletons at age 8 years enrolled during 2011-14	PFOA, PFOS, PFNA, PFHxS and metabolome were measured in serum samples.	PFAS: online SPE-HPLC-MS/MS; Metabolome: Orbitrap LC–HRMS (Dionex Ultimate 3000, Q-Exactive, Thermo Scientific)	In the RPLC C18-negative mode, 17, 63, 47, and 29 features (confidence levels 1 to 4) were associated with serum PFOA, PFOS, PFNA, and PFHxS concentrations; In the HILIC-positive mode, 18, 253, 76, and 39 features (confidence levels 1 to 4) were associated with serum PFOA, PFOS, PFNA, and PFHxS concentrations, respectively, at FDR <20%.	Pathways associated with all four types of PFAS included arginine, proline, aspartate, asparagine, and butanoate metabolism.
(Hu et al. 2019)	California, US	397 pregnant women (an average age 25 years) enrolled during 1959-67. Fifty of these women	PFOS, EtFOSAA and metabolome were measured in serum samples.	PFAS: online SPE-HPLC-MS/MS; Metabolome: Orbitrap LC–HRMS (Q-	PFOS was associated with 34 features, and EtFOSAA was associated with 49 features. Features with confidence level 1 associated with PFOS included betaine, carnitine, creatine, adrenochrome, aminoisobutyric	Among the 12 associated pathways, the two common pathways that EtFOSAA and PFOS shared were glycine, threonine, alanine and serine

Publication	Location	Study design and sample size	PFAS and non-targeted metabolome measures[a]	Analytical platform for PFAS and metabolome	Significant metabolomic features associated with PFAS (FDR <0.05[b], confidence levels 1 to 3)	Significant pathways (p for pathway enrichment test <0.05)
		developed breast cancer.		Exactive HF (Thermo Fisher)).	acid, arginine, asparagine, clupanodonyl carnitine, glutamate, hexadecenoyl carnitine, histidine, linoelaidyl carnitine, lysine, N1-Methyl-4-pyridone-5-carboxamide, N6,N6-dimethyl-L-lysine, peptide 2-(3-carboxy-3-aminopropyl)-L-histidine, phosphoserine, tetracosapentaenoyl carnitine.	metabolism, and urea cycle/amino group metabolism.
(Alderete et al. 2019)	Urban Los Angeles, California, US	Hispanic overweight youths (8-14 years) with a family history of T2D enrolled during 2001-12 and were followed at 1-to-3-yr.	PFOA, PFOS, PFHxS were measured in plasma at baseline and metabolome were measured in plasma in both baseline and follow-up.	PFAS: LC-HRMS Metabolome: Orbitrap LC–HRMS (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific)	Plasma PFOA, PFOS and PFHxS were associated with 149, 298, and 17 metabolite features (confidence levels 1 to 4), respectively, at FDR <20%.	24 metabolic pathways were associated with PFAS exposure, including significant alterations of lipids (*e.g.*, glycosphingolipids, linoleic acid), and amino acids (*e.g.*, aspartate and asparagine, tyrosine, arginine and proline).
(Maitre et al. 2018)	Catalonia and Basque Country, Spain	750 pregnant women from Sabadell (2004-08) and Gipuzkoa (2006-08) with an average age 30 years.	PFHxS, PFNA, PFOA, PFOS in first-trimester serum and metabolome were measured in the first and the third trimester serum samples.	PFAS: HPLC-MS/MS Metabolome: NMR spectroscopy (Bruker Biospin, Rheinstetten, Germany)	In Gipuzkoa cohort, pregnanolone-3G, acetone, succinate were prospectively associated with PFHxS, while alanine, glycine, 3-hydroxybutyrate/3-aminoisobutyrate were prospectively associated with PFOA. However, these associations were not replicated in the Sabadell cohort.	n/a
(Salihovic et al. 2019)	Uppsala, Sweden	965 older adults (aged 70 years) with equal gender distribution enrolled during 2001-04	PFHxS, PFOS PFHpA, PFOA, PFNA, PFUdA and metabolome were measured in overnight fasting plasma samples	PFAS: UPLC-MS/MS; Metabolome: UPLC-Q-ToF-MS (Waters Corporation, Milford, USA)	Lipid related metabolites (*e.g.*, glycerolipids, glycerophospholipids, and fatty acids), and amino acid and purine related metabolites were associated with PFAS. Features of confidence level 1 included L-proline, monoacylglycerol (16:1), phosphatidylcholine, uric acid.	Eight PFAS-associated metabolites mapped to human metabolic pathways were enriched in glycerophospholipid metabolism (p =0.004), linoleic acid metabolism (p = 0.04), and α-linoleic acid metabolism (p = 0.07).
(Wang et al. 2017a)	Shandong, China	181 male adults (median age 34) without metabolic diseases	PFOA, PFOS and metabolome were measured in serum samples.	PFAS: LC-MS; Metabolome: UPLC/ Orbitrap-MS (Thermo, USA)	10 identified features (confidence levels 1 to 3) associated with PFAS included α-CEHC, arachidonic acid, D-glucurono-6,3-lactone, hypoxanthine, oxoglutaric acid, pyroglutamic acid, BH4, xanthine, DAHA, hydroxybutyric acid, at p<0.05.	[d] The lipid metabolism, xenobiotic detoxifying, antioxidation and the NO signal pathways may be affected by PFAS exposure.
(Lu et al. 2019)	Hubei, China	40 workers (mean age 45) and 52 controls (mean age 50) from the general population enrolled in 2017	PFBA, PFOA, PFBS, PFHxS, PFOS, 6:2 Cl-PFESA and metabolome were measured in overnight fasting plasma samples.	PFAS: HPLC-MS/MS; Metabolome: UPLC-Q Orbitrap MS system (Thermo, USA) GC-MS (Agilent 7890B/ 5977A Series GC/ Mass Selective Detector system)	Features of confidence level 1 associated with PFAS included pyroglutamic acid, ornithine, hypoxanthine, DL-2-aminooctanoic acid, γ-CEHC, 3-hydroxyoctanoic acid, C18:2-CN, C18:1-CN, sebacic acid, methionine sulfoxide, GPC, azelaic acid, myo-inositol, piperine at p<0.05.	[d] PFAS were associated with lipid metabolism, amino acids metabolism, purine metabolism, inositol metabolism, retinol metabolism, and metabolism of alkaloids and their derivatives.

[a] Four studies used fasting samples (Schillemans, 2020; Chen, 2020; Salihovic, 2019; Lu, 2019). Six studies (Jin, 2020; Li, 2020; Kingsley, 2019; Hu, 2019; Maitre, 2018; Wang, 2017) used non-fasting samples. Alderete, 2019: Fasting and post-challenge plasma samples were collected at baseline but unclear which was for PFAS or metabolomics assays.

[b] The significance level for metabolomic features in these studies was false discovery rate (FDR)<0.05 if not specified in the table.

[c] Li, 2020: The significance of associations from PLS regression was assessed by permutation on both community member and sample labels. Instead of feature-level associations, the individual features are organized as members of communities, and the associations are tested at community level across exposome and metabolome.

[d] Lu, 2019 conducted pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, while Wang, 2017 did not specify the tools used for pathway analysis.

Abbreviations: α-CEHC: alpha-carboxyethyl hydroxychromanol; BH4, Tetrahydrobiopterin; 6:2 Cl-PFESA, 6:2 chlorinated polyfluorinated ether sulfonate; CN, acylcarnitine; DAHA: deoxyarabinohexonic acid; EtFOSAA, 2-(N-Ethyl-perfluorooctane sulfonamido) acetic acid; FDR, false discovery rate; γ-CEHC: gamma-carboxyethyl hydroxychroman; GC, gas chromatography; HRMS: high-resolution mass spectrometry; GPC, glycerophosphocholine; HPLC, high performance liquid chromatography; LC, liquid chromatography; LysoPC: lysoophosphatidylcholine; MS, mass spectrometry; NMR, nuclear magnetic resonance; NO, nitric oxide; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutanesulfonic acid; PFDA, perfluorodecanoic acid; PFHpA, perfluoroheptanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; QToF: quadrupole time-of-flight; RPLC, reversed phase liquid chromatography; SPE, solid-phase extraction; T2D, type 2 diabetes; UPLC: ultra-pressure liquid chromatography.

Table 2.

A summary of studies reporting the metabolomic associations related to both PFAS exposures and health outcomes.

Publication	Health outcomes	Main findings
(Schillemans et al. 2020)	Type 2 diabetes (T2D)	PFAS were associated with two groups of lipid species with opposite relations to T2D risk: glycerophospholipids were correlated positively with PFAS and were inversely associated with risk for T2D, while diacylglycerols were correlated positively with both PFAS and risk for T2D.
(Chen et al. 2020)	Cardiometabolic outcomes (OGTT measures, body fat and lipid profiles)	Increased lipolysis and fatty acid oxidation were contributing to the biological mechanisms linking PFAS exposure and impaired glucose metabolism among young adults.
(Jin et al. 2020)	Non-alcoholic fatty liver disease (NAFLD)	Each interquartile range increase of PFHxS was associated with increased odds for liver fibrosis, lobular inflammation, and higher NAFLD activity score. A cluster of children with nonalcoholic steatohepatitis was characterized by increased PFAS levels and altered metabolite patterns including higher plasma levels of phosphoethanolamine, tyrosine, phenylalanine, aspartate and creatine, and decreased plasma levels of betaine.
(Alderete et al. 2019)	Changes in glucose homeostasis from a baseline visit to a 1-to-3-yr visit among adolescents at risk of T2D	Higher PFAS exposure was associated with dysregulation of several lipid and amino acid pathways and longitudinal alterations in glucose homeostasis in Hispanic youth.

Abbreviations: OGTT, oral glucose tolerance test; PFAS, per- and polyfluoroalkyl substances; PFHxS, perfluorohexane sulfonic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate.