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ABSTRACT

The wide-spread environmental pollutants per- and polyfluoroalkyl substances (PFAS) have repeatedly been associated with elevated serum cholesterol in humans. However, underlying mechanisms are still unclear. Furthermore, we have previously observed inverse associations with plasma triglycerides. To better understand PFAS-induced effects on lipid pathways we investigated associations of PFAS-related metabolite features with plasma cholesterol and triglyceride concentrations.

We used 290 PFAS-related metabolite features that we previously discovered from untargeted liquid chromatography-mass spectometry metabolomics in a case-control study within the Swedish Västerbotten Intervention Programme cohort. Herein, we studied associations of these PFAS-related metabolite features with plasma cholesterol and triglyceride concentrations in plasma samples from 187 healthy control subjects collected on two occasions between 1991 and 2013.

The PFAS-related features did not associate with cholesterol, but 50 features were associated with triglycerides. Principal component analysis on these features indicated that one metabolite pattern, dominated by glycerophospholipids, correlated with longer chain PFAS and associated inversely with triglycerides (both cross-sectionally and prospectively), after adjustment for confounders. The observed time-trend of the metabolite pattern resembled that of the longer chain PFAS, with higher levels during the years 2004–2010.

Mechanisms linking PFAS exposures to triglycerides may thus occur via longer chain PFAS affecting glycerophospholipid metabolism. If the results reflect a cause-effect association, as implied by the time-trend and prospective analyses, this may affect the general adult population.

1. Introduction

The omnipresent environmental pollutants per- and polyfluoroalkyl substances (PFAS) are a group of persistent fluorinated chemicals used for years in different consumer products (*e.g.* coatings or fire-fighting foam) that have been associated with elevated total cholesterol levels in humans (ATSDR, 2018; EFSA, 2020; Steenland et al., 2020; Sunderland et al., 2019). Several efforts are being undertaken, *e.g.* in the Horizon 2020 project Human Biomonitoring for Europe (www.hbm4eu.eu), to clarify pathways potentially involved in such associations. Meanwhile, our previous research found an inverse association of PFAS with triglycerides, but not with cholesterol (Donat-Vargas et al., 2019c). Both cholesterol and triglycerides are relevant factors implicated in cardiovascular disease development (Farnier et al., 2021; Lewington

Abbreviations: LC-PFAS, longer chain per- and polyfluoroalkyl substances; MCs, metabolite components; PFAS, per- and polyfluoroalkyl substances; PPARs, peroxisome proliferator-activated receptors; SC-PFAS, shorter chain per- and polyfluoroalkyl substances.

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et al., 2007). The exact mechanisms involved in PFAS-related lipid perturbations are still unknown. They may interfere with fatty acid oxidative or endocrine pathways (Jensen and Leffers, 2008; Kar et al., 2017; Sheng et al., 2018), via activation of several transcription factors involved in glucose and lipid metabolism (*e.g.* peroxisome proliferator-activated receptors (PPARs) (Rosen et al., 2017; Vanden Heuvel et al., 2006), constitutive androstane receptor (Abe et al., 2017), endocrine receptors (Benninghoff et al., 2010) and pregnane X receptor (Bijland et al., 2011)).

Metabolomics, the study of small molecules involved in metabolic processes, is a useful tool for investigating the biochemical effects of exposures on metabolism (Kim et al., 2016; Kingsley et al., 2019; Vineis et al., 2013). Thus far, metabolomic-based studies have shown associations between PFAS (most prominent for long chain PFAS) and metabolites mainly from lipid pathways (Jin et al., 2020; Salihovic et al., 2019). In our previous study on PFAS and type 2 diabetes, employing the "meet-in-the-middle" approach for molecular epidemiology (Vineis et al., 2013), we found 290 metabolite features associated with PFAS using a random forest-based methodology (Schillemans et al., 2020). Of these, several also associated with type 2 diabetes and many were lipid-related metabolites, mainly glycerophospholipids and diacylglycerols (Schillemans et al., 2020).

In the present study, we aimed to gain more insight in PFAS-induced effects on lipid pathways, thus we investigate associations between the previously identified PFAS-related metabolite features and plasma concentrations of total cholesterol and triglycerides. We used two repeated measurements approximately 10 years apart among participants from the Swedish Västerbotten Intervention Programme (VIP) cohort. During the time under study (1991–2013), the exposure to several PFAS through diet increased for a certain period, parallelled by increases in plasma (National Food Agency, 2017). This allowed us to explore whether PFAS-related metabolite patterns and lipid concentrations followed PFAS exposure temporal trends, which would be expected in case of true cause-effect associations.

2. Materials and methods

2.1. Study participants

Data was used from a nested case-control study within the VIP cohort, which included participants that had donated overnight fasting blood samples to the biobank on two occasions, on average 10 (SD ± 4.4) years apart. The selection of the study participants is described elsewhere (Donat-Vargas et al., 2019a, 2019c). In brief, the Västerbotten Intervention Programme was initiated in 1985 and invited all residents in Västerbotten County in Northern Sweden for health screening and blood sampling when they turned 40, 50 and 60 years. Participation rate exceeded 56%, being often around 70%, and 90.5% of participants also donated blood samples (Norberg et al., 2010). Incident diabetes cases, identified in the DiabNorth register (participation rate 70%) and diagnosed according to WHO recommendations and analysis of autoantibodies (Rolandsson et al., 2012), were individually matched with controls based on age, sex and date of blood draw. This study included baseline data between 1991 and 2003 and follow-up data between 2000 and 2013. PFAS and metabolites were measured in 374 participants at baseline and follow-up (187 diabetes case-control pairs). All 374 participants were included in an initial exploratory phase where PFAS-related metabolite features were discovered. In the present study, that investigated associations between PFAS-related metabolite features and plasma lipid levels, the analysis was restricted to repeated measures of the 187 control subjects.

Participation information from questionnaires included attained education, smoking status, physical activity (Cambridge index for physical activity) and body mass index (BMI). Information on a healthy diet score (1–25 score, Baltic Sea diet (Kanerva et al., 2014; Shi et al., 2018a)) and alcohol consumption (g/day) were based on a validated food frequency questionnaire (Johansson et al., 2001, 2002; Nettleton et al., 2013). Informed consent was obtained from all participants and the study protocol was approved by the regional ethics review boards in Umeå and Uppsala, Sweden (Dnr, 2013/414-31, 2014/147-32M and 2014/011).

2.2. Targeted LC-MS PFAS measurements and lipid measurement

PFAS concentrations were measured by targeted liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) as described in detail elsewhere (Koponen et al., 2013). Baseline and follow-up concentrations of perfluorododecane acid (PFDoA), perfluorotridecane acid (PFTrA), perfluorotetradecane acid (PFTA), Perfluorohexanoic acid (PFHxA), perfluoroheptane sulfonate (PFHpS), perfluoroheptane acid (PFHpA) and perfluorodecane sulfonate (PFDS) were <limit of quantitation (LOQ) in all subjects (LOQ was 0.30 ng/ml except for PFHpS that was 1.0 ng/ml) and they were not analysed further. Quantifiable concentrations were detected for perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnDA). Concentrations below the LOQ were set to the LOQ/2 = 0.075 ng/mL for 16% of PFDA and 39% of PFUnDA in the control subjects alone.

Plasma cholesterol and triglyceride concentrations were measured in the same fasting samples (8-h overnight) as used for PFAS measurements. Reflotron was used up to September 2009 and afterwards measurements were performed in the clinical chemical laboratory (Donat-Vargas et al., 2019c).

2.3. Untargeted LC-MS metabolite profiling

Data acquisition, corrections and normalization of the metabolomics dataset is described elsewhere (Brunius et al., 2016; Shi et al., 2017). In brief, untargeted LC-quadrupole time of flight (qTOF)-MS metabolic profiling was performed on baseline and follow-up plasma samples (Shi et al., 2018a). A constrained randomization was applied to keep case-control sample pairs and baseline/follow-up samples within the same batch. De-proteinised fasting heparin plasma samples were analysed by LC-qTOF-MS (Agilent Technologies, United States) on reverse phase and hydrophilic interaction liquid chromatography columns in both positive and negative ionization modes. The MassHunter Acquisition B.04.00 software (Agilent Technologies) was used for data acquisition. Raw data were converted to mzXML format and deconvolution was performed ('XCMS' R package) (Shi et al., 2017). Measurement drift was adjusted for using quality control samples ('batchCorr' R package) (Brunius et al., 2016). Features were retained if they passed QC test (CV < 0.3) in at least five out of the eight analytical batches (Supplemental Material in Shi et al., (2018a, b) (Shi et al., 2018b)). Missing values were replaced with values selected at random from a normal distribution between 0 and the lowest measured peak intensity for each metabolite feature under the assumption of missing not at random due to low concentration. Poorly retained lipids in hydrophilic interaction chromatography (retention time <70s) were removed, as these were better represented in reverse phase. We use the term metabolite feature to refer to a molecular entity with a unique mass-to-charge ratio and retention time as measured by LC-MS. We previously putatively annotated six metabolite features as possible PFAS (Schillemans et al., 2020), which were excluded from the metabolomics dataset in order not to bias data analytical models.

2.4. PFAS-related metabolite features

To obtain the PFAS-related metabolite features, we first derived PFAS exposure patterns, using orthogonally (varimax) rotated principal component analysis (PCA) on individual square root-transformed PFAS, scaled to unit variance as described in detail elsewhere (Schillemans

et al., 2020). Two PFAS patterns (longer chain PFNA, PFDA and PFUnDA *vs* shorter chain PFOS, PFOA, PFHxS) were obtained through manual inspection of the first two PFAS component loadings (eigenvalue>1), referred to as longer (LC-PFAS) and shorter chain PFAS (SC-PFAS). Using the metabolomics data as predictors and each of the individual square root-transformed PFAS concentrations and the two PFAS patterns as target variables, the data were processed using a random forest model within a repeated double-cross validation framework incorporated with unbiased variable selection (R package MUVR (Shi et al., 2019b), https://gitlab.com/CarlBrunius/MUVR). Modelling performance was assessed by permutation analysis (n = 50, p < 0.001) (Lindgren et al., 1996). The robustness of the discovered PFAS-associated metabolite features was tested by a series of similar analyses, stratified by sex and measurement occasion. Only metabolite features present in both the overall model and in one of the stratified models were selected, thereby excluding features likely originating from differences related to sex or baseline *vs* follow-up measurement (corresponding to sex and measurement occasion adjustment). Parameters and results are discussed in Supplemental Material in Schillemans et al., (2020) (Schillemans et al., 2020).

2.5. Associations of PFAS-related metabolites with total cholesterol and triglycerides

In order to further reduce the number of metabolite features and include only features related to potential lipid disturbances, associations of the selected PFAS-related metabolite features (square-root transformed and standardized) with plasma total cholesterol and triglycerides were assessed. For this, we used repeated measures analyses at the two timepoints (baseline and follow-up) by generalized estimating equations (GEE). Here we excluded subjects using cholesterol-lowering medication (n = 13) and those with missing lipid data (n = 3 for cholesterol and n = 25 for triglycerides). All analyses were adjusted according to matching factors (age, gender and sample year) as well as education, smoking status, physical activity, alcohol intake and healthy diet score. Resulting p-values from all models were adjusted for multiple testing (false discovery rate (FDR) < 0.05). Furthermore, the hypergeometric test was used to investigate whether chance could have produced the number of PFAS-related metabolite features that were also associated with lipids (p < 0.05).

To gain further insight into the connections between PFAS exposures, PFAS- and lipid-related metabolite features and lipid outcomes, we used the triplot approach for visualization (Schillemans et al., 2019). We first aggregated the PFAS-related metabolite features associated with lipids from the GEE model (FDR<0.05) into metabolite components (MCs) using PCA with oblimin rotation. The triplot visualized 1) the loadings of individual PFAS- and lipid-related metabolite features in the MCs, 2) correlations of participants' MC scores with PFAS exposures and BMI (partial Spearman correlation adjusted for the same set of covariates and averaged over baseline and follow-up) and 3) associations of participants' MC scores with total cholesterol and triglycerides (GEE adjusted for the same set of covariates). To illustrate the temporal trends we visualized the sex- and birthyear-standardized medians of LC-PFAS, relevant MC-scores and lipid concentrations for each calendar year over the whole study period (1991–2013) and additionally adapted data from the Swedish National Food Agency on PFDA, PFNA and PFUnDA intake per calendar year (National Food Agency, 2017).

Stratification by sex was included to test for potential differences in associations between men and women. We also assessed the consistency of the associations with the cardiometabolic risk factors in a prospective model (using the average MC scores at baseline and follow-up, covariates at baseline and lipid outcomes at follow-up). Finally, we validated the associations in a larger dataset from the same cohort that lacked PFAS measurements but had available baseline metabolomics data and cardiometabolic risk factors measurements (Shi et al., 2018b). The validation data had similar characteristics as the cohort under study. Statistical analyses were performed using R software version 3.4.3 (R Core Team, Vienna, Austria) (RCoreTeam, 2017).

2.6. Metabolite annotation

To annotate the metabolites, we followed the Metabolomics Standards Initiative (MSI) reporting criteria for the confidence level. Available auto-MS/MS data was matched to literature based on accurate mass and product ion spectrum (level 2) (Hanhineva et al., 2014; Shi et al., 2018a, 2019a). Other metabolite features associated with PFAS and triglyceride concentrations were putatively annotated for compound class based on m/z (mass tolerance <20 ppm) matched against online databases (level 3). Unknown compounds were presented as "analytical mode _ m/z @ retention time" (level 4).

3. Results

Table 1 describes the study characteristics for the controls. For further details and PFAS concentrations see previously published data (Donat-Vargas et al., 2019b, 2019d). Out of the 290 PFAS-related metabolites previously discovered (Schillemans et al., 2020), 50 associated with triglycerides in the GEE model (FDR<0.05; Fig. 1, Supplemental Table S1), which is more than what would be expected due to chance (n_{chance} = 1.3, $p_{hypergeometric}$ < 0.001, Supplemental Table S2). In contrast, only 2 PFAS-related metabolites were associated with total cholesterol (FDR <0.05, Fig. 1, Supplemental Table S1), which could be related to chance findings and were therefore not considered further (n_{chance} = 0.8, $p_{hypergeometric}$ = 0.18, Supplemental Table S2).

Among the 50 metabolite features, several belonged to lipid classes of diacylglycerols and glycerophospholipids and we furthermore identified carnitine 13:1, carnitine 13:0, trimethoxycinnamic acid, oxo-tetradecadienoic acid and 7-ketocholesterol (Supplemental Table S3). Both carnitines, trimethoxycinnamic acid and oxo-tetradecadienoic acid correlated positively with PFAS and associated inversely with triglycerides, whereas 7-ketocholesterol correlated negatively (although not strongly) with PFAS and associated positively with triglycerides (Supplemental Tables S1 and S4). The PCA performed on the 50 PFAS- and triglyceride-related metabolite features revealed three MCs with eigenvalues >3 that together represented 37% of the variance. MC 1 had high loadings from several glycerophospholipids, correlated strongly

Table 1
Participant characteristics of study control subjects by sampling occasion after exclusion of those on lipid-lowering medication.

	Study Controls Baseline (n = 187) (1991–2003)	Study Controls Follow-up (n = 174) (2001–2013)
Characteristics		
Female	86 (46%)	81 (47%)
Age [yrs.]	46 (6)	56 (6)
Sample year	1996 (3)	2006 (3)
Body mass index [kg/m2]	25 (3)	26 (4)
Education >12 yrs.	135 (72%)	129 (74%)
Smoking status		
Current	40 (21%)	22 (13%)
Former	71 (38%)	78 (42%)
Physical activity		
Inactive	107 (57%)	91 (52%)
Healthy diet [1–25 score]	11 (3)	13 (4)
Alcohol consumption [g/day], %		
0.1–5	123 (66%)	109 (63%)
5.1–15	49 (26%)	53 (30%)
>15	5 (3%)	6 (3%)
Laboratory analyses		
Total cholesterol [mmol/l]	5.5 (1.1)	5.4 (1.1)
Triglycerides [mmol/l]	1.3 (0.6)	1.4 (0.7)

Note: Continuous variables are shown as mean (standard deviation) and categorical variables are shown as counts (percentage).

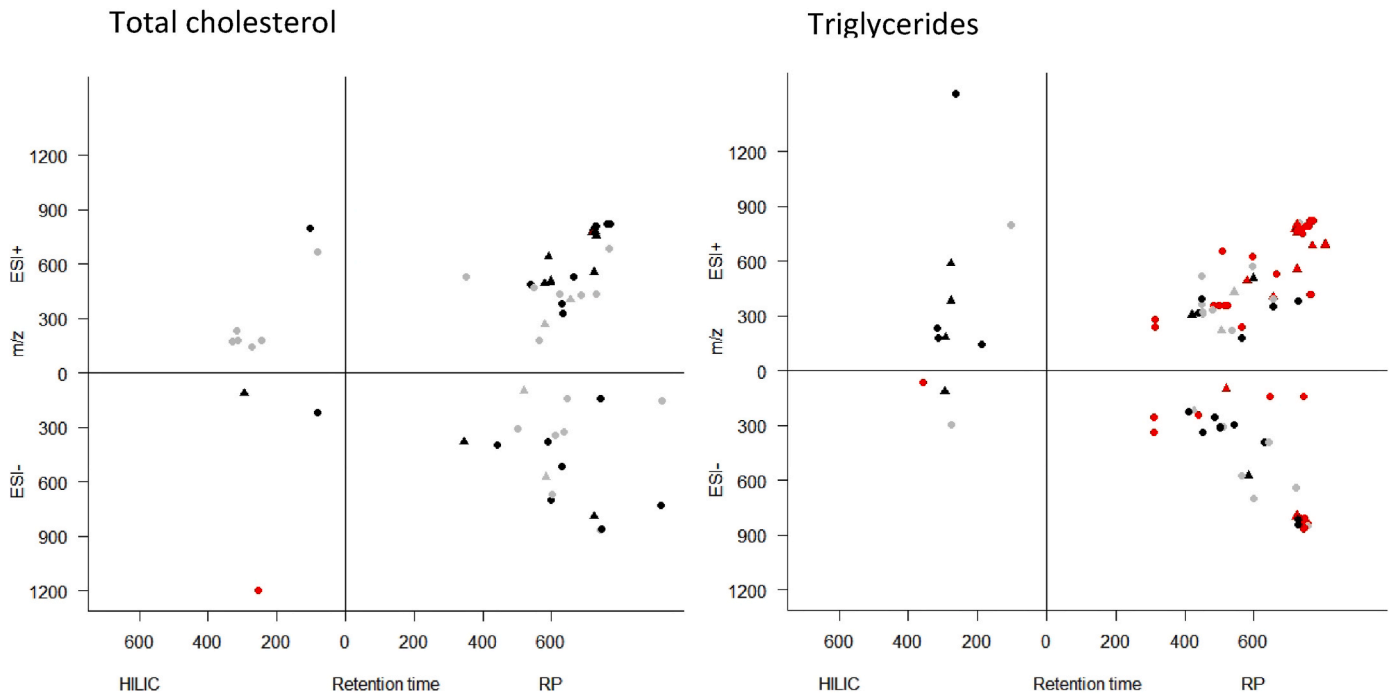


Fig. 1. Plots of PFAS-related features negatively (round) or positively (triangle) associated with total cholesterol (n = 358) (left) and triglycerides (n = 336) (right), adjusted for sex, age, sample year, education, smoking status, physical activity, alcohol intake and healthy diet score. Colors indicate FDR <0.05 (red), $p < 0.05$ (black) and $p < 0.1$ (grey), whereas features with $p > 0.1$ are not shown. Features are separated by column and ionization mode, as well as ordered by retention time and mass-to-charge ratio (m/z). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with LC-PFAS and associated inversely with triglycerides and borderline with total cholesterol (Fig. 2, Supplemental Table S5). Neither MC 2 nor MC 3 correlated strongly with PFAS, nor associated with total cholesterol. MC 2 had high loadings from trimethoxycinnamic acid and 3 other unidentified features and associated inversely with triglycerides. MC 3 had high loadings from three diacylglycerols, correlated positively with BMI and fatty fish intake and associated with higher triglyceride concentrations.

The results for triglycerides from the adjusted model (age, gender, sample year, education, physical activity, smoking status, alcohol consumption and healthy diet score) did not differ qualitatively from the model adjusted only for age, sex and sampling year. When stratified by sex, the associations for triglycerides were observed in both sexes, whilst the inverse associations for total cholesterol were stronger for women in MC 1 and MC 3 (Supplemental Figure S1). Associations for total cholesterol in the prospective analysis lowered to null, whereas those for triglycerides were stronger in MC 2 (inversely) and in MC 3 (positively) (Supplemental Figure S1). The validation data indicated similar significant inverse associations for MC 1 and triglycerides but mainly null-associations for total cholesterol and the other two MCs (Supplemental Figure S1).

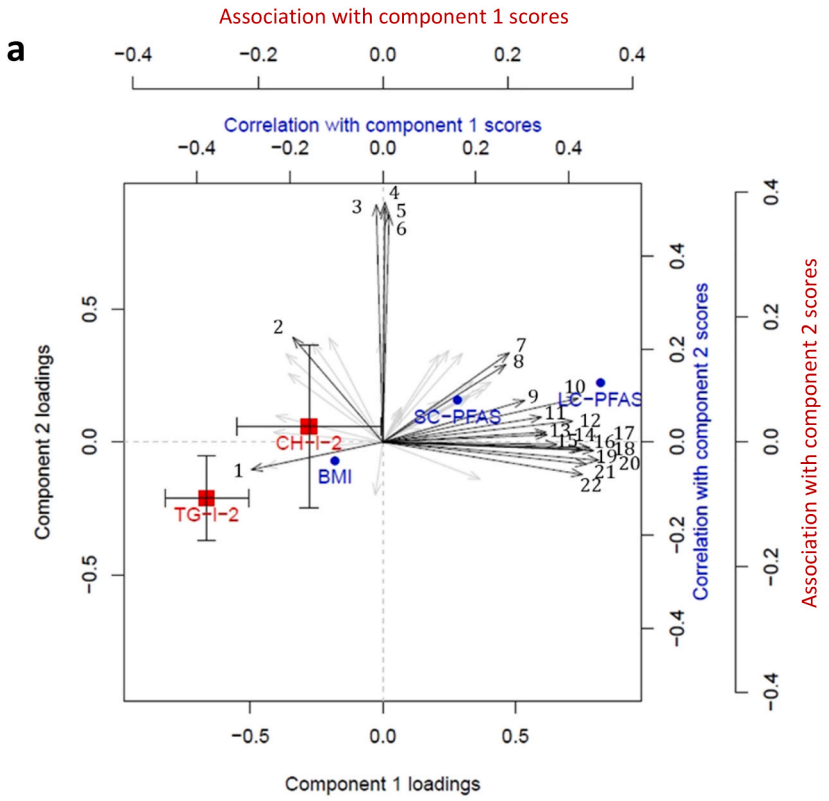
In order to further explore the evidence for a causal association between PFAS on the one hand and MC 1 and triglycerides on the other, we graphically visualized the time-trend for PFNA, PFDA and PFUnDA intake (from the Swedish National Food Agency) as well as for LC-PFAS and MC 1 scores and triglyceride concentrations in blood of study subjects over the period 1991–2015. We observed that the plasma LC-PFAS and MC 1 scores, but not the triglyceride concentrations, followed the fluctuations in dietary exposures to LC-PFAS (Fig. 3).

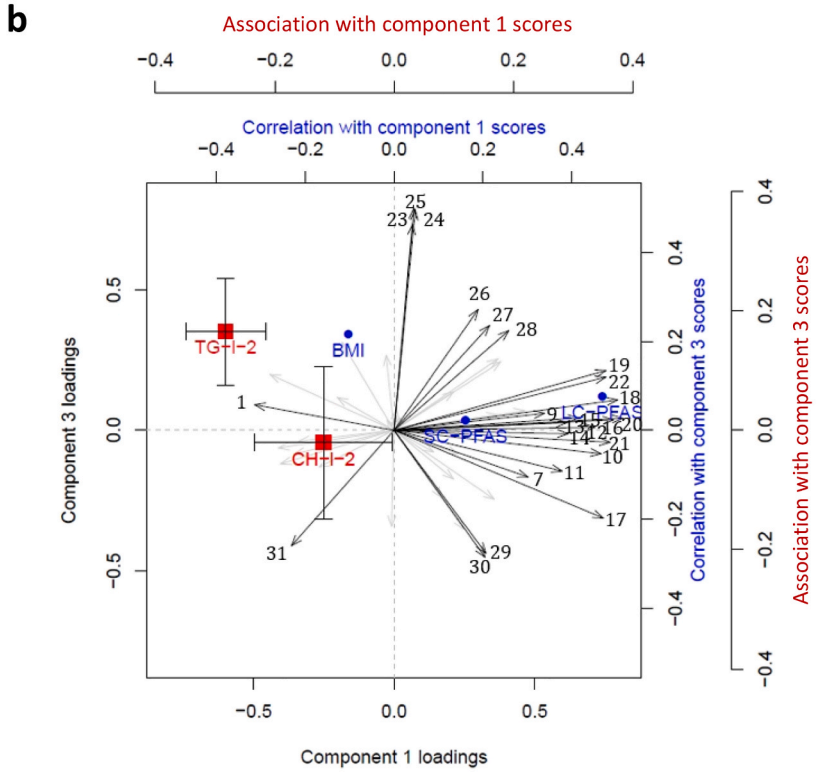
4. Discussion

The present study was conducted to investigate associations of PFAS-related metabolite features with circulating concentrations of total cholesterol and triglycerides. Although there is largely consistent evidence for an association between PFAS and elevated total cholesterol concentrations, we found no molecular signatures supporting this link in our study. In contrast, we found 50 PFAS-related metabolite features that associated with triglyceride concentrations. One of the metabolite components, obtained from a PCA on these features, was dominated by glycerophospholipids and correlated positively with LC-PFAS, but inversely with triglycerides.

The present study builds on a previous publication which - without involving metabolomics - also observed null-associations between PFAS and total cholesterol but inverse associations with triglycerides (Donat-Vargas et al., 2019c). However, although the causality and biological mechanisms are not fully clarified yet, most observational epidemiological studies – in contrast to animal studies (Pouwer et al., 2019; Seacat et al., 2002) – are supportive of associations between PFAS exposures and elevated total cholesterol, (EFSA panel on Contaminants in the Food Chain CONTAM et al., 2018; EFSA, 2020; Steenland et al., 2020), with some exceptions (Château-Degat et al., 2010; Donat-Vargas et al., 2019c). In addition, several of the PFAS- and triglyceride-related metabolite features similarly associated with type 2 diabetes in our previous study, based on the same study participants (Schillemans et al., 2020), which is plausible considering the connection between plasma triglycerides and type 2 diabetes (Parhofer, 2015; Wu and Parhofer, 2014).

To facilitate the interpretation of results involving several metabolites, we aggregated the selected PFAS- and triglyceride-related metabolite features into three MCs. The first component was dominated by glycerophospholipids and it correlated positively with LC-PFAS and associated inversely with triglycerides, which remained stable in the prospective and the validation data assessments. This suggests that LC-PFAS exposures (*i.e.* PFNA, PFDA and PFUnDA) are associated with lower triglyceride concentrations through perturbations of several glycerophospholipids. This is biologically plausible since triglyceride and glycerophospholipid metabolism are closely connected as they share precursors and several enzymatic steps in their biosynthesis (Aung et al., 2013). Similar observations regarding PFAS exposure, reduced





c

1) DG_RP554.48@724.74
2) RP492.30@580.42
3) RP297.08@313.05
4) Trimethoxycinnamic acid
5) RN255.08@311.53
6) RN339.04@311.52
7) GPL_RP791.55@748.58
8) GPL_RP820.56@771.76
9) GPL_RN860.58@746.44
10) GPL_RP792.58@760.42
11) RP415.77@760.96
12) GPL_RP766.57@735.87
13) GPL_RP819.59@769.71
14) GPL_RP764.54@731.32
15) GPL_RP748.52@740.94
16) GPL_RN808.55@726.15
17) GPL_RN836.58@740.42
18) GPL_RN810.56@747.27
19) GPL_RN834.56@740.87
20) GPL_RN836.58@755.11
21) RP_415.76@764.54
22) GPL_RN810.56@732.92
23) DG_ RP682.53@769.90
24) DG_ RP684.55@805.11
25) DG_ RP689.51@805.34
26) Carnitine 13.0
27) GPL_RN864.57@743.36
28) GPL_RP820.59@761.63
29) RP_357.21@524.13
30) RP_357.22@524.47
31) GPL_RP775.54@727.80

d

	MC1	MC2	MC3
PFOS baseline	0.23	0.12	0.04
PFOA baseline	0.08	0.04	0.06
PFHxS baseline	0.17	0.06	0.08
PFDA baseline	0.4	−0.03	0.04
PFNA baseline	0.38	0.1	0.11
PFUnDA baseline	0.41	0.11	0.03
LC−PFAS baseline	0.45	0.04	0.05
SC−PFAS baseline	0.12	0.08	0.04
PFOS follow−up	0.23	0.19	0.07
PFOA follow−up	0.2	0.1	0.01
PFHxS follow−up	0.23	0.09	0.05
PFDA follow−up	0.42	0.26	0.11
PFNA follow−up	0.4	0.12	0.14
PFUnDA follow−up	0.41	0.23	0.02
LC−PFAS follow−up	0.49	0.22	0.1
SC−PFAS follow−up	0.2	0.1	0
BMI baseline	−0.17	0.01	0.19
BMI follow−up	−0.04	−0.09	0.24
Fatty fish baseline	0.09	0.04	0.24

Fig. 2. Triplot with metabolite components from 50 PFAS- and triglyceride-related metabolite features. Arrows indicate metabolite loadings in the components. Metabolite component correlations are shown for PFAS exposures and BMI (blue circle). Repeated measures associations (B-coefficient and 95% CI) are shown for triglycerides (n = 336) and total cholesterol (n = 335) (red square). Analyses were adjusted for age, gender, sample year, education, physical activity, smoking status, alcohol consumption and healthy diet score. Panels represent **a)** components 1 and 2, **b)** components 1 and 3, **c)** labels of metabolite features (only shown for 31 metabolites with loadings >0.4), and **d)** correlations per measurement. **Abbreviations:** Cholesterol (CH), Diacylglycerols (DG), Glycerophospholipids (GPL), Longer chain PFAS (LC-PFAS), Shorter chain PFAS (SC-PFAS) and Triglycerides (TG). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

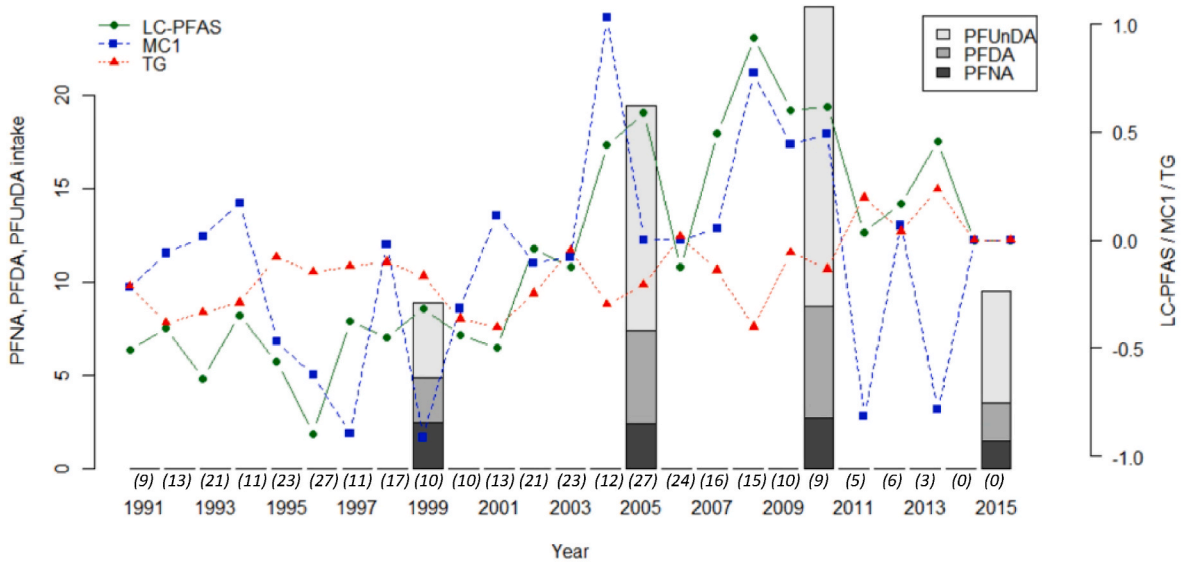


Fig. 3. Temporal trends displayed for longer chain (LC-)PFAS levels, metabolite component (MC) 1 scores and triglyceride concentrations in the Västerbotten Intervention Programme cohort complemented by information on temporal trends of PFNA, PFDA and PFUnDA intake in Sweden. Presented as sex- and birthyear-standardized LC-PFAS (green points and line), MC 1 (blue squares and dash), and triglycerides (red triangles and dots) in 336 samples from 172 control subjects from 1991 to 2013. Number of samples in each calendar year indicated in parenthesis in italics. PFNA, PFDA and PFUnDA intake is presented as median per calendar year in ng/day (indicated by grey bars) [adapted from Swedish Market Basket Survey (2015) performed by the National Food Agency, rapportserie nr 26/2017]. **Abbreviations:** Cholesterol (CH), Metabolite Component 1 (MC 1), Longer chain PFAS (LC-PFAS), Shorter chain PFAS (SC-PFAS) and Triglycerides (TG). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

triglycerides and upregulations of glycerophospholipids were found in a mouse study, albeit for PFOS (Li et al., 2021). Although there is also a possibility for false-positive or -negative findings, residual confounding or reverse causation, several of our results dispute this. First, the robust findings in the hypergeometric test and validation data indicate that chance findings and overfitting are limited. Second, residual confounding is likely to be only minor as we adjusted for several important lifestyle factors which hardly impacted model estimates. Nevertheless, PFAS have affinity for phospholipids and proteins which may be relevant for PFAS partitioning and accumulation (De Silva et al., 2021), thus reverse causation for the association between PFAS and glycerophospholipids could be plausible.

However, a 'natural experiment' of increasing LC-PFAS diet and plasma concentrations occurred during the study period which could shed more light on causality (Fig. 3). There are two main observations following this 'natural experiment':

1) The intermediate outcome (MC 1) follows the temporal trends of the LC-PFAS exposure, which could be observed if **A)** the exposure affects molecular pathways and this alters the outcome (causal) or if **B)** the outcome is in fact the exposure (*i.e.* metabolite features in MC 1 consist of PFAS metabolites).
2) The outcome (triglycerides) does not follow the temporal trends of the LC-PFAS exposure, which could be observed if **C)** the outcome affects internal LC-PFAS exposure (*i.e.* reverse causation *e.g.* via partitioning mechanisms), **D)** a molecular pathway affects both LC-PFAS and outcome (*i.e.* confounding) or if **E)** the outcome is also regulated by other factors or molecular pathways and the effect of the exposure is too small to be graphically visualized in a time-trend.

For MC 1 we consider that a direct reflection of the exposure **(1B)** is unlikely since correlations between the specific metabolite features and PFAS were not high and we did not identify any features as PFAS compounds, thus strenghtening the assumptions of a causal association. For triglycerides, we consider a dilution effect **(2E)** is most likely, based on the observed prospective associations, but we cannot fully exclude reverse causation (2C) or confounding (2D).

The second and third components were dominated by unannotated features and diacylglycerols, respectively, and the second associated inversely whilst the third associated positively with triglycerides. However, neither component two or three correlated strongly with PFAS, which may indicate that these features may be related to potential confounding factors, such as diet (EFSA panel on Contaminants in the Food Chain CONTAM et al., 2018; Donat-Vargas et al., 2019c). However, only the third MC correlated moderately positively with self-reported fish intake and adjustment for healthy diet score had merely marginal effect on the estimates of the MCs with triglycerides or total cholesterol. In addition, we annotated trimethoxycinnamic acid and oxo-tetradecadienoic acid, which have been reported to reflect fish exposure (Shi et al., 2018a, 2019a), but these loaded only weakly in MC 3. Thus, the underlying biological phenomena reflected in these components remain largely unknown and may reflect potential unmeasured confounding.

One of the biological pathways suggested for PFAS-induced lipid perturbations is through activation of transcription factors such as PPARs, which play important roles in the regulation of lipid metabolism (Pouwer et al., 2019). The hypothesis of PFAS-induced perturbations in lipid pathways is strengthened in the current study by the fact that PFAS-related features associated with triglycerides could mainly be annotated to classes of lipids (*i.e.* diacylglycerols and glycerophospholipids). These results are similar to previous metabolomics studies which have also associated PFAS exposures to circulating glycerophospholipids and diacylglycerols (Alderete et al., 2019; Hu et al., 2019; Jin et al., 2020; Kingsley et al., 2019; Salihovic et al., 2019). Moreover, we identified two carnitines (*i.e.* 13:0 and 13:1) that were positively correlated to PFAS exposure and inversely associated with triglycerides and cholesterol. Carnitines are involved in transporting long-chain fatty acids to the mitochondrial matrix for energy production and they are upregulated by PPARα activation (Song et al., 2010). The carnitine shuttle was found to be positively associated with PFOS in a previous metabolomics study on PFAS exposure (Hu et al., 2019). The inverse association of these carnitines with lipid outcomes in the present study is supported by a previous nutrition study relating diet to cardiovascular disease markers (*e.g.* cholesterol and triglycerides) (Tovar

et al., 2017). Interestingly, carnitine transport is also important for fatty acid oxidation (Longo et al., 2016) and we also found an oxo fatty acid (oxo-tetradecadienoic acid) positively correlated with PFAS and inversely associated with triglycerides. Some oxo fatty acids have been shown to activate PPARα and PPARγ and decrease triglyceride levels (Goto, 2019; Kim et al., 2012). Additionally, we found negative correlations between PFAS exposure and 7-ketocholesterol, a product of a reaction between cholesterol and oxygen radicals found in arterial plaque (Anderson et al., 2020), potentially indicating that PFAS mitigates these reactions.

Interestingly, although total cholesterol was not significantly associated with a PFAS-related metabolite profile (hypergeometric test $p > 0.05$), it had a borderline inverse association with the first PFAS- and triglyceride-related MC. This could indicate that the triglyceride-related metabolite features when aggregated also reflect cholesterol metabolism. This association was most apparent among women, indicating possible gender differences in the molecular associations between PFAS exposure and physiology. Similar gender differences were previously observed in studies investigating PFAS and lipid concentrations (Jain and Ducatman, 2019). Nevertheless, caution is required for these interpretations as associations between MCs and total cholesterol were not significant in prospective assessments nor in the validation data.

Several limitations and strengths regarding the untargeted metabolomics methods and study design have been discussed in detail in our previous paper (Schillemans et al., 2020), but some should be mentioned here as well. The three components (MCs) captured only 37% of the variance of all PFAS- and triglyceride-related metabolite features. The remaining variance may be attributed to random variability, but we cannot exclude that other potentially relevant associations may have been overlooked. In addition, unfortunately not all metabolite features could be annotated since not all features resulted in MS/MS fragmentation of sufficient quality. However, several features could still be annotated to compound class. An important strength of this study is the longitudinal design and repeated measurement modelling which allowed for robust association testing. In addition, we also investigated the robustness of the partial correlations and GEE approach for repeated measures within the meet-in-the-middle framework and contrasted it to a change-in-change analysis, also adapted to capitalise on repeated measures (Dunder et al., 2022; Fitz-Simon et al., 2013), with similar results. We therefore conclude that observed results were not obtained by modelling artefacts. Furthermore, the associations between the first PFAS- and triglyceride-related metabolite component, dominated by glycerophospholipids, and triglycerides was strengthened by both prospective analysis and independent validation data. These results may not be generalizable to people exposed to higher levels of PFAS and future studies to validate these results in independent cohorts, preferably with wide variability in PFAS exposures (*e.g.* polluted areas), from prospective studies and including other omics data such as transcriptomics or proteomics, could shed more light on pathways and causality.

In conclusion, we did not find evidence for associations of PFAS-related metabolite feature patterns with total cholesterol. Instead, we discovered metabolites that reflected PFAS exposure and were associated with triglycerides, which validated our previous findings of inverse associations of PFAS in plasma with triglycerides (Donat-Vargas et al., 2019c), and these metabolites may mediate PFAS-related effects on plasma triglycerides. These results highlight the importance of investigating associations between PFAS and triglycerides in addition to the relationship between PFAS and total cholesterol. These results suggest that associations between PFAS and triglycerides may be an effect of longer chain PFAS affecting glycerophospholipid metabolism. If the results reflect a cause-effect association, as implied by the time-trend and prospective analyses, this may affect the general adult population.

Author statement

T. Schillemans: Methodology, Formal analysis, Visualization, Writing – original draft. **I.A. Bergdahl:** Funding acquisition, Methodology. **K. Hanhineva:** Resources, Data curation, Methodology. **L. Shi:** Data curation, Software. **C. Donat-Vargas:** Data curation, Supervision. **J. Koponen:** Resources. **H. Kiviranta:** Resources. **R. Landberg:** Funding acquisition, Methodology. **A. Åkesson:** Conceptualization, Funding acquisition, Methodology, Supervision. **C. Brunius:** Conceptualization, Funding acquisition, Methodology, Software, Supervision. **All authors:** were involved in reviewing the manuscript and approved the final version.

Declaration of competing interest

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

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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