Multi-omics in research: epidemiology, methodology, and advanced data analysis

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Chapter 7

PFAS concentrations are associated with a cardio-metabolic risk profile: findings from two population cohorts

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1 ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are widely used and persistent chemicals leading to ubiquitous and constant exposure. Although high PFAS levels have been associated with hypercholesteremia and cardiovascular disease, the levels of PFAS and associations with metabolic risk markers in general population samples are not fully characterized. We thus aimed to assess the associations between perfluorooctaneic acid (PFOA), perfluorooctane sulfonic acid (PFOS), and perfluorohexanesulfonic acid (PFHxS) and a range of metabolites as well as clinical lipid measurements. For the analysis, we used participants with complete clinical lipid, metabolite and PFAS measurements from the NEO (n= 586) and Rhineland Study (n= 1,962). The metabolites were measured with nuclear magnetic resonance by Nightingale and were mainly comprised of lipoprotein characteristics. Using linear regression analyses, we quantified age-, sex- and education-adjusted associations of PFOA, PFOS, and PFHxS (Rhineland Study only) with clinical lipid measurements and metabolites (n= 224).

In line with previous research, both studies confirmed that PFAS, particularly PFOS and PFHxS, were associated with higher clinically measured LDL and cholesterol concentrations. We uniquely showed that this was characterized by higher concentrations of total lipid, cholesterol and phospholipid content in LDL particles in particular. We also showed an interaction effect of age on the majority of associations, where the effect of PFAS was stronger in younger people (≤ 54 years). Thus, our results show that even low PFAS concentrations are associated with an unfavorable lipid profile in the general population. This emphasizes the need for further regulation of PFAS substances.

2 INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS), colloquially known as the 'forever chemicals', are man-made chemicals that have been widely used in many industrial processes and products since the 1950s (1). These chemicals are persistent and resilient in nature, allowing them to circulate in water sources and become widespread around the globe (2-9). High PFAS levels may be caused by contamination in the vicinity of PFAS-producing factories, but also by the use and breakdown of PFAS-containing products—such as fire extinguishers, non-stick cooking pans, certain food packaging and pesticides (6, 10). Contact with this contaminated environment or these products may lead to exposure in humans via media such as drinking water, inhalation or dermal exposure. Exposure may also be indirect, for example through the diet (6). Given the persistence of PFAS, human exposure is continuous and ubiquitous.

Soon after the introduction of PFAS production, several health concerns were raised. Direct high exposure of PFAS has been associated with various adverse health outcomes, including obesity, kidney disease, cancer, thyroid disease, hypercholesteremia, dyslipidemia, liver damage, reduced antibody response to vaccination, and a higher risk of severe course of COVID-19 infection (4, 7-9). Previous research has associated PFAS with changes in the immune system, proteome (11), hormones (12), and metabolome (11, 12). Furthermore, a 2020 report by the European Food Safety Authority (EFSA) indicated that the risks associated with PFAS were even more severe than previously believed. They also reported that low PFAS levels, previously thought to be within the safe limits, could pose a health risk as well (8, 13, 14). However, while high PFAS exposure has been the focus of the majority of PFAS studies, much remains unclear about the effects at low exposure in the general population (6, 15).

Specific mechanisms through which PFAS exert their effects are unclear. Previous studies have shown that PFAS have been most consistently associated with changes in lipid metabolism, particularly higher cholesterol levels. Thus far, those studies have only considered traditional, composite lipid measurements, such as total, HDL and LDL cholesterol, and it remains unclear how PFAS affect the deeper metabolic and lipoprotein profiles (4).

The health concerns from PFAS exposure resulted in the classification of certain PFAS as persistent pollutants that require regulation. Specifically, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been subjected to growing restrictions (7, 16). However, despite the increasing scrutiny, many PFAS species remain unregulated and even PFOA and PFOS are not yet fully banned in the European Union (17). Moreover, despite efforts to reduce and discontinue PFOA and PFOS production in the Netherlands (17, 18) and Germany (19), levels and exposure remain an issue (13, 14, 20).

Whilst PFAS exposure thus continues, consequences of regular exposure to PFAS in the general population and the metabolomic effects of PFAS remain understudied. Here, we aimed to evaluate the association of PFAS levels in the general population with metabolites and lipoproteins using clinical lipid measurements and targeted metabolomics. To improve generalizability and robustness of the results, we performed the analysis in two study populations: the Netherlands Epidemiology of Obesity (NEO) study (n= 586) and the Rhineland study (n= 1,962).

3 METHODS

3.1 Study Populations

3.1.1 Netherlands Epidemiology of Obesity Study

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study of individuals aged 45–65 years, with an oversampling of individuals who are overweight or have obesity. Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m2 or higher, living in the greater area of Leiden (in the West of the Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited, irrespective of their BMI. Recruitment of participants started in September 2008 and was completed at the end of September 2012. In total, 6,671 participants have been included. Participants were invited to come to the NEO study center of the LUMC for one baseline study visit after an overnight fast. A blood sample of 108 mL was taken from the participants after an overnight fast of at least 10 hours (21). In the current study, only participants from the Leiderdorp sub-population with untargeted metabolomic data were included, leading to a sample size of 599 individuals. Among these individuals, 4 did not have targeted metabolomics data and 9 were excluded due to measurement errors, leading to a final sample size of n= 586 (**Figure 1**).

3.1.2 Rhineland Study

The Rhineland Study is an ongoing community-based cohort study located in two geographically defined areas in Bonn, Germany. Participation is possible by invitation only. To be able to participate, participants had to be above 30 years of age at baseline and have sufficient command in German to provide informed consent. The current analysis was based on the first 5,000 subsequent participants whose plasma was analyzed for PFAS levels (n= 4,469), Nightingale metabolites (n= 1,982) and whose education level was known, resulting in a final analytical sample of 1,962 participants (**Figure 1**). Of these, 1,805 participants had complete data on LDL, HDL, total triglyceride, and cholesterol levels, which were used for the additional analysis on clinical lipid measurements.

3.2 PFAS measurements

Relative PFAS concentrations were acquired on the untargeted Metabolon™ Discovery HD4 platform at Metabolon Inc. (Durham, North Carolina, USA). In brief, this process involves four independent ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS/MS) platforms (22, 23). It uses two positive ionization reverse phase chromatography, one negative ionization reverse phase chromatography, and one hydrophilic interaction liquid chromatography negative ionization (23). In NEO, the PFOA and PFOS levels were quantified from fasting state serum samples, while PFOA, PFOS and perfluorohexanesulfonic acid (PFHxS) concentrations were obtained from fasting plasma samples in the Rhineland Study. PFAS were quantified as relative concentrations. To ensure normality and comparability across studies, we log-transformed and Z-standardized all PFAS concentrations for the subsequent analyses.

3.3 Metabolites and lipoprotein measurements

Total serum levels of cholesterol, triglycerides, LDL, and HDL were measured in NEO as described in previous work (21). In the Rhineland Study, serum levels of cholesterol, triglycerides, LDL and HDL were measured with routine measurements at the University Hospital in Bonn.

Fasting serum (NEO) and plasma (Rhineland Study) metabolite levels were quantified using the Nightingale (Nightingale Health Ltd, Helsinki, Finland) nuclear magnetic resonance untargeted metabolomic platform. This platform quantified 224 metabolites and metabolite ratios. The data predominantly contains detailed lipoprotein lipid information. Additionally, absolute concentrations of various other metabolites, including amino acids, free fatty acids and ketone bodies, were quantified (24).

3.4 Assessment of covariates

In both the Rhineland Study and NEO, questionnaire and food frequency questionnaires were used to collect demographic and lifestyle information, including smoking history (yes/no), alcohol intake (g/day), and education (low/middle/high). In the Rhineland Study, missing values for smoking were imputed based on HD4-measured cotinine levels in the blood according to the method described by St Helen et al (25). To assure the quality of the data, alcohol values belonging to participants reporting an overall improbable caloric intake (<600 or >8,000) were excluded, as per the method of Galbete et al. (26).

The International Standard Classification of Education 2011 (ISCED) (27) was used to standardize education across both studies. Participants' education level was reclassified as low (lower secondary education or below), middle (upper secondary education to undergraduate university level) or high (postgraduate university study) in both studies. In the NEO study, the education status of two participants was missing and set to "low".

3.5 Statistical Analysis

3.5.1 Imputation of Missing Metabolite Values

A total of 224 metabolites were measured on the targeted metabolomics Nightingale platform. These metabolites had missing values ranging from 0.3% to 9%. Missing values were set to 0 where the levels were believed to be below detection. Missingness due to other reasons was imputed using a previously described pipeline (28). Accordingly, imputed datasets were generated using multiple imputation. To ensure normality, all metabolites were log-transformed after adding 1 to account for 0s.

3.5.2 Linear Regression Models and Meta-Analysis

Multiple linear regression models were performed to associate the log-transformed Nightingale and clinical lipid measurements (outcomes) with the log-transformed and Z-standardized PFAS concentrations (exposures). In model 1, we adjusted for sex (women/men), age (years) and education (low/middle/high). As PFAS accumulate during the lifespan and may have a different effect with increasing age, model 2 additionally included a multiplicative interaction term between age and the PFAS substances. We also assessed the possible difference between men and women in model 3 by including a sex-interaction term (29). If the interaction estimate was significant, a stratified analysis was performed on the basis of sex or the median age (54 years). For ease of comparability in the figures, analyses were additionally run on the Z-standardized metabolite levels after the log transformation. To test whether the associations were dependent on traditional risk behaviors, we also ran an analysis adjusting for alcohol intake, smoking, and body mass index (BMI). Lastly, we assessed the robustness of our results by performing a sensitivity analysis where we truncated extreme outlier values (>5 standard deviations) in the metabolite levels.

A meta-analysis was performed on the metabolites and stratified if the age-interaction term was significant. To improve the assessment of the confidence intervals and account for heterogeneity for the two studies, we conducted a meta-analysis using both the fixed and the random model.

3.5.3 Multiple testing correction

As the Nightingale measurements are inherently highly correlated, we used the method described by Li and Ji (30) to calculate the effective number of independent variables. Accordingly, the number of independent metabolite variables was estimated at 66 (70 including clinical lipid measurement outcomes) in both studies. Thus, we considered a p-value < 0.0007 (0.05/70) as statistically significant.

All analyses were performed using R (31) v4.1.0 (2021-05-18) in NEO and v4.0.5 (2021-03-31) in the Rhineland study. The meta-analysis was conducted using the *rmeta* package. Figures were created using the *ggplot 2*(32) R package.

4 RESULTS

4.1 Population characteristics

Participants in the Rhineland Study had a median age of 54 years (range: 30 – 89), consisted of 57% women, had an average BMI of 25.8, and were relatively highly educated (**Table 1**). NEO participants had a median age of 54 years (range: 45 – 66) and were composed of 52.6% women. Alcohol intake in the Rhineland Study was higher than in NEO. Smokers made up 13.7% and 11.3% of the Rhineland and NEO study, respectively. PFOA and PFOS was measured in all 599 participants of the NEO study. In the Rhineland Study, PFOA, PFOS, and PFHxS were measured in 1967, 1950, and 1964 out of 1967 participants, respectively.

4.2 Linear Regression

4.2.1 Overview

The role of sex and age in the association between PFAS and clinical lipid measures and metabolites was assessed by interaction analyses. No consistent difference in the associations between men and women was found. Contrastingly, we found a significant age-interaction for the majority of the associations (**Table 2, Table 3, Table 4, Table 5**).

4.2.2 PFOA

No associations were found between PFOA and clinically measured lipids in model 1 without an interaction term (**Table 2**, **Table 3**). In the age-interaction model, PFOA was associated with higher LDL levels in the Rhineland Study. The age-interaction term was negative, indicating a smaller effect with increasing age. Contrastingly, analyses after age stratification showed that the effect of PFOA on LDL levels in the Rhineland study was smaller in the younger age group (≤54 years) compared to the older group (>54 years) (**Table 3**). In NEO, PFOA was nominally associated with increased cholesterol concentrations, but not associated after adjustment for multiple testing (**Table 2**).

In model 1, we found 3/224 and 2/224 metabolites associated with PFOA levels in the NEO and Rhineland studies respectively (**Table 4**, **Table 5**). In NEO, these metabolites were related

to lipids in the small HDL particles while in the Rhineland study they were associated with cholesterol in HDL 3, sphingomyelins, and albumin. After adding the PFOA-age interaction term, 3/224 and 1/224 PFOA associations showed a significant age-interaction in the NEO and Rhineland studies respectively. In contrast to model 1, these all related to LDL and cholesterol content in NEO, while only the LDL size was associated in the Rhineland Study. In NEO, the age-interaction term indicated a stronger effect in younger people, which the age-stratified analysis confirmed, while the age-stratified analysis showed conflicting results in the Rhineland Study (**Table 4**, **Table 5**).

4.2.3 PFOS

In model 1, no associations were found between PFOS and the 4 clinical lipid measurements in either study. After the addition of the PFOS-age interaction term, we found that PFOS was associated with elevated levels of cholesterol and LDL in both NEO and the Rhineland Study, where the age-interaction term indicated a weaker effect with increasing age. This was echoed by the stratified analysis, which showed a stronger effect in the younger age group (≤54 years) in both studies (**Table 2**, **Table 3**).

In model 1, PFOS was associated with 3/224 metabolites in the NEO study, while no significant associations were found in the Rhineland Study (**Table 4**, **Table 5**). All significant associations in the NEO study were related to fatty acids. The age-interaction analysis revealed 53/224 and 80/224 associations between the PFOS-age interaction term and metabolites in the NEO and Rhineland studies, respectively (**Table 4**, **Table 5**). In both studies, these were primarily related to LDL, VLDL, IDL, apolipoprotein B (apoB), and fatty acids. In the Rhineland Study, PFOS was also associated with valine, phosphatidylcholines, albumin, phosphoglycerides and sphingomyelins. Overall, the age-interaction term indicated a weaker effect with increasing age. This was confirmed by the age-stratified analysis, which showed a stronger effect in the younger age group (≤ 54 years) compared to the older group (>54 years) (**Table 4**, **Table 5**).

4.2.4 PFHxS

PFHxS was measured only in the Rhineland study and was not associated with any of the clinical lipid measurements in model 1. In the age-interaction model, PFHxS was associated with higher cholesterol levels, the effect of which was weaker with older age. In the age-stratified analysis, the association between PFHxS and cholesterol was indeed stronger in the younger group (≤54 years) (**Table 3**).

PFHxS was associated with 40/224 of the metabolites in model 1. PFHxS was generally associated with increased levels of cholesterols, LDLs, fatty acids, albumin, and apolipoprotein A (apoA) (**Table 5**). On the other hand, PFHxS was associated with a decrease in the amino acid phenylalanine only. When adding the age-interaction term, we observed an age effect in 8/224 metabolites, namely fatty acids, cholesterols, phosphoglycerides, and phosphatidylcholines. Although the age-interaction term always showed a weakening of the effect with age, the age-stratified analysis only confirmed this for the fatty acids and phosphoglycerides. Contrastingly, the effect of PFHxS was stronger in the older group (>54 years) for cholesterols (**Table 5**).

4.3 Sensitivity analyses

When additionally adjusting for smoking, alcohol consumption, and BMI in the complete data in the NEO (n= 586) and the Rhineland studies (n= 1,733), the number of associations generally increased slightly for both the model with and without the age-interaction (**Supplementary**

Table 1). The overall results, however, remained consistent for PFOA and PFOS. Results for PFHxS in model 1 did not change substantially either. Contrastingly, we found an additional 15 significant age-interactions when adjusting for the aforementioned covariates. New associations mainly comprised of VLDL, and LDL and apoB (**Supplementary Table 1**).

A separate sensitivity analysis using truncated outliers of PFAS concentrations was also performed. In the Rhineland Study, the number of significant associations increased for PFOA (n= 9), PFOS (n= 68), and PFHxS (n= 61) (**Supplementary Table 1**). Generally, these associations spanned the categories of apolipoproteins, cholesterols, glycerides and phospholipids, HDL, IDL, LDL and VLDL. PFAS concentrations thus showed an even stronger consistent association with cholesterols and LDL concentrations and composition in particular. In the age-interaction model, we found fewer age-interactions after accounting for outliers for PFOS (n= 28). Remaining associations included apoB, cholesterols, fatty acids, LDLs and VLDLs. In the NEO study, the results of the sensitivity analyses remained largely consistent with the main results (**Supplementary Table 1**).

4.4 Meta-analysis

In the meta-analysis, we found that the heterogeneity (I2) was low in all analyses, indicating that the two populations are similar. Furthermore, the fixed and random effect model estimates and confidence intervals overlapped and were in the same direction. Hence, the fixed effect model was an appropriate method for this analysis.

In the meta-analysis for model 1, PFOA was associated with higher levels of clinically measured cholesterol and HDL, as well as a number of metabolites. The latter included the total concentrations of cholesterols, LDL, VLDL, IDL, HDL, and the lipid content of these lipoproteins. Moreover, PFOA was associated with higher levels of amino acids, fatty acids, and glycerides. Similarly, increased levels of PFOS were associated with higher clinically measured cholesterol and LDL, as well as various metabolomics measurements. Specifically, PFOS was associated with increases in the levels of LDL, IDL and VLDL, as well as their lipid content. Furthermore, it was associated with cholesterols, amino acids, fatty acids and HDL content, specifically that of the very large HDL particles (**Supplementary Table 2).**

In the age-stratified meta-analysis, we showed similar results to the study-specific analysis. Specifically, the different PFAS were associated with higher levels of clinical lipids and some metabolites (**Supplementary Table 3**). The PFOA-associated metabolites belonged to the groups of small VLDL, omega fatty acids and the size of LDL. On the other hand, PFOS was associated with valine, various fatty acids, albumin, phosphoglycerides, apoB, and cholesterols as well as the composition and concentration of IDL, LDL, and VLDL. Unlike the meta-analysis for model 1, no associations with any HDL metabolites or clinical HDL measurements were found. When comparing the different age-groups, we found more significant associations in the younger age group (age ≤54) compared to the older age group (>54). Furthermore, fixed effect estimates tended to be stronger in the younger group.

5 DISCUSSION

5.1 Summary

In this study, we investigated the association of three PFAS substances with clinically measured lipid biomarkers and a wide range of metabolites (n= 224) in the general population. By combining

findings from the NEO Study (n= 586) and the Rhineland Study (n= 1,962), we report common and clinically relevant effects of PFAS on lipid metabolism. In particular, PFAS molecules were associated with higher levels of clinically measured LDL and cholesterol, which was confirmed by the association with lipoprotein metabolites. Specifically, PFOS and PFHxS were associated with a metabolomic profile characterized by increased levels of apoB, phosphoglycerides, total lipids, fatty acids, and the lipid content in LDL, IDL, and VLDL. Meta-analyses showed a similar trend across the populations with small heterogeneity, further strengthening our findings. Thus, we interpret these data to indicate that even low PFAS levels in the general population have a detrimental effect on lipid metabolism.

5.2 Widespread PFAS exposure in the Netherlands and Germany

PFAS production, and thus exposure, in both Germany and the Netherlands started in the second half of the last century. In the Netherlands, production of PFAS substances began at the DuPont/ Chemours plant in Dordrecht in 1967 (33). Although the production of the so-called legacy PFAS (PFOA and PFOS) was slowly phased out in 2012 and replaced by "GenX", both surface and ground water, soil, vegetation, fish, and stock animals in the area remain highly contaminated. In addition, contamination in surface and drinking water was detected across the western regions of the Netherlands (34). The National Institute for Public Health and the Environment (RIVM) has reported that the Dutch population is likely ingesting PFAS levels above the recommended safe levels via food and water (14). Moreover, they advised against consuming fruits or vegetables grown from gardens within a 1 km radius of the Dordrecht Chemours plant and from the Westerschelde area downstream of the plant (35). Accordingly, the RIVM has concluded that the levels of PFAS in the Netherlands are highly concerning and require further research (14). In Germany, PFAS have been produced since 1968 at the Chemiepark Gendorf (CPG) (36). Therefore, contamination is widespread in the ecosystem and PFAS are detectable in drinking water (37, 38). Specifically, areas along the Rhine, Ruhr and Moehne rivers are marked as high exposure locations (37). For example, contaminated paper sludge caused high PFAS levels in the drinking water in Rastatt county in the Baden-Wuerttemberg state (37) and the town of Arnsberg in the state of North Rhine-Westphalia (39). Importantly, PFAS levels are detectable in the groundwater of most of the provinces and in all of the soil samples (19). It is thus clear that PFAS exposure is widespread in both Germany (19, 20, 37, 39) and the Netherlands (14, 34, 35, 40). Indeed, despite the production of legacy PFAS being slowly phased out over the years, PFAS levels were detectable in nearly all included participants from the NEO and the Rhineland studies.

5.3 PFAS levels associated with metabolic profile of increased risk of cardiovascular disease

Here, we showed that even low concentrations of PFAS were associated with a distinctive lipid profile. Overall, PFAS substances were associated with an increase in clinically measured total LDL and cholesterol. Further investigation using metabolomics revealed that higher PFAS levels were characterized by elevations in cholesterol and lipid content in LDLs and VLDLs of all sizes. We also found associations with apoB, fatty acids, phosphoglycerides, IDL, and phospholipids. Previously, a higher lipid content of lipoproteins was implicated in cardiovascular disease (CVD) (41), while higher levels of fatty acids and apoB were consistently associated with myocardial infarction (42). Other studies have linked a similar metabolomic profile to a higher risk of cardiometabolic diseases such as CVD (43), hypertension (44), type 2 diabetes (DM2) (45), and non-alcoholic fatty liver disease (46). Therefore, our results suggest that PFAS exposure may increase the risk of cardiometabolic outcomes by impacting the lipoprotein composition. Of note, we

found that PFHxS, one of the often-used substitutes of PFOA and PFOS (6, 47), similarly affected metabolite concentrations, as well as lipoprotein composition and concentrations.

Importantly, the abovementioned results for PFOA and PFOS were further strengthened by our meta-analysis, which showed that PFAS might have clinically relevant effects in the European population. For example, every one standard deviation increase in PFOS in the younger group was associated with a 0.03 increase in log-transformed LDL levels across the two populations, which is equal to an LDL increase of 0.1 mmol/L (CI: 0.04 – 0.20). Recommended thresholds for LDL in patients with CVD or DM2 are <1.8 mmol/L and <2.6 mmol/L, respectively. As such, we show that even general population levels of PFAS might have a clinically relevant effect.

5.4 Effects of PFAS are partially dependent on Age

We also reported a significant age-interaction for the majority of metabolite PFOA and PFOS associations, which generally showed a weakening of the effect in older individuals. On the other hand, PFHxS showed a weaker age effect. Finding a weakening of the effect of PFAS is therefore not unexpected, as other competing causes might have a higher relative importance than PFAS exposure. In people within the age range of 40-50, the absence of medication or other lifetime-accumulated exposures might therefore mean that PFAS have a higher relative impact on their lipid levels. Alternatively, the time of exposure might also impact the effect and explain the difference. Indeed, children have been reported to suffer from more severe effects than adults (48).

In the Netherlands, pre-determined cut-offs for safe levels of PFAS were recently used to conclude that the extremely high PFAS levels in the Westerschelde were no cause for concern (35). However, our results indicate that in the general population low levels of PFAS are associated with a detrimental lipid profile. Moreover, associations were robust and even increased in number in the sensitivity analysis. Taken together with previous literature, stricter regulations are required for all PFAS substances. Furthermore, due to the persistent nature of PFAS and their recirculation in the environment, there is a need to actively remove these chemicals from the environment—methods for which are under development (49).

5.5 Strengths and limitations

Our main limitation stems from the cross-sectional nature of our data. As such, we cannot establish a causal link between the PFAS exposures and the metabolites. Nonetheless, our data shows a clear link between PFAS and a cardio-metabolic risk profile across two European populations. These results are in line with previous findings, and as such, should be taken into consideration when assessing the risk and required regulation of PFAS across the whole population. Other limitations include the use of different sample media for the measurement of PFAS and Nightingale metabolites in NEO versus the Rhineland Study, as well as the use of relative, rather than absolute, PFAS concentrations. Despite these limitations, the inclusion of two European countries demonstrates a consistent and robust association with PFAS levels. Furthermore, we evaluated their relation to a detailed lipid profile and a large variety of metabolites.

6 CONCLUSION

In conclusion, our results expand on previous findings by showing a clear link between a harmful lipid profile and PFAS concentrations across different study populations, even at low PFAS levels. We report an association with increased LDL and total cholesterol as well as apoB and lipid content in LDL, VLDL, and IDL lipoproteins. The effect generally weakened with increasing age, indicating that PFAS exposure is particularly detrimental at a younger age. The combination of the well-documented persistence of PFAS and their harmful effects ensures that exposure to these substances is an enduring public health challenge. Thus, there is a clear need for further studies in general populations, as well as regulation and efforts to reduce environmental PFAS levels.

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8 STATEMENTS AND DECLARATIONS

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Competing Interests
R. Li-Gao is a part-time clinical research consultant for Metabolon, Inc. All other authors have no relevant financial or non-financial interests to declare.

Author Contributions
T.O. Faquih: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft Preparation, Visualization; **E.N. Landstra.:** Conceptualization, Methodology, Formal Analysis, Writing – Original Draft Preparation, Visualization; **R. de Mutsert**: Project Administration, Resources, Funding Acquisition, Writing – Review and Editing; **R. Noordam and D. van Heemst**: Funding acquisition, Writing – Review and Editing; **F.R. Rosendaal.**: Study design, Funding acquisition, Conceptualization; **A. van Hylckama-Vlieg and K.W. van Dijk**: Conceptualization, Supervision, Writing – Reviewing and Editing; **D.O. Mook-Kanamori:** Conceptualization, Methodology, Resources, Writing – Reviewing and Editing, Funding Acquisition, Supervision; **M.M.B. Breteler:** Conceptualization, Resources, Writing – Reviewing and Editing, Data Curation, Funding Acquisition, Supervision.

All authors read and approved the final manuscript

Data availability Rhineland Study data used for this manuscript are not publicly available due to data protection regulations. Access to data can be provided to scientists in accordance with the Rhineland Study's Data Use and Access Policy. Requests for additional information and/or access to the datasets can be send to RS-DUAC@dzne.de.All authors had full access to their respective study data and take responsibility for the integrity of the data and the accuracy of the analysis.

Ethics approval
The NEO study was approved by the medical ethical committee of the Leiden University Medical Centre (LUMC) and all participants gave their written informed consent.

The ethics committee of the medical faculty of the University of Bonn approved the undertaking of the Rhineland Study and it was carried out according to the recommendations of the International Council for Harmonisation Good Clinical Practice standards.

Consent to participate
Written informed consent was acquired from all participants per the Declaration of Helsinki in both the NEO and Rhineland Study.

Tables and Figures

Figure 1: **Overview of study population selection for NEO (A) and the Rhineland Study (B)**

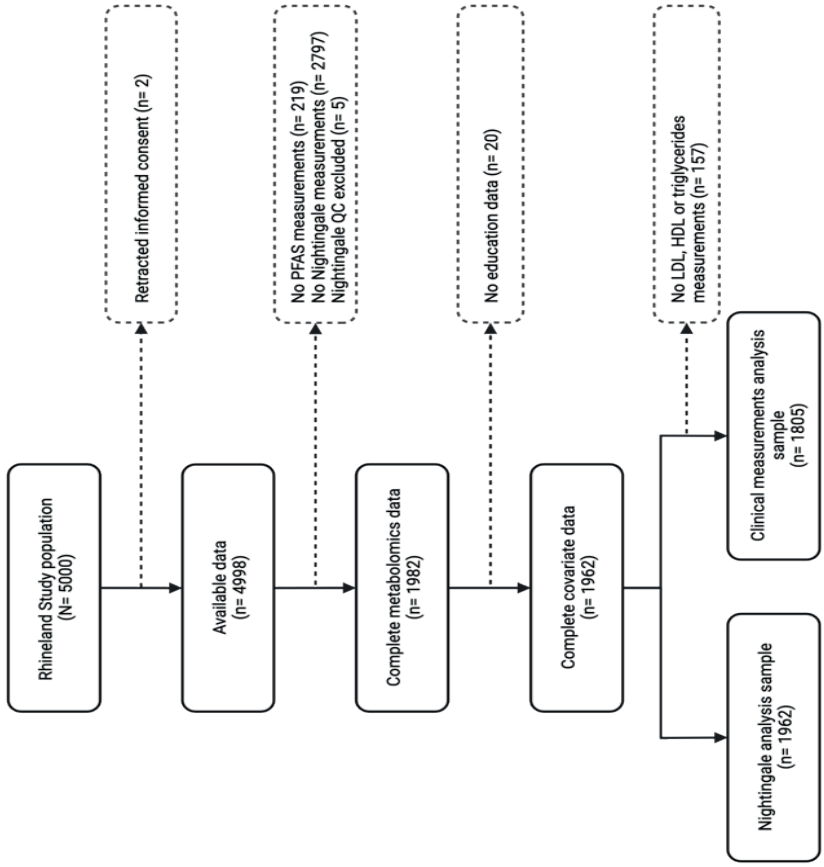

Table 1: **Population characteristics** *of the NEO and Rhineland Study*

Characteristic[†]	NEO Study			Rhineland Study		
	All (n= 586)	*Women (n= 308)*	*Men (n= 278)*	*All (n= 1,962)*	*Women (n= 1,104)*	*Men (n= 858)*
Age (years (SD))	55.8 (5.9)	56.1 (6.2)	55.7 (5.7)	54.6 (14.1)	54.5 (13.7)	54 (14)
Sex (Women (%))	308 (52.6)			1104 (56.3)		
PFOA (mean (SD))	1.07 (0.50)	1.11 (0.51)	1.04 (0.49)	1.11 (0.53)	1.10 (0.62)	1.13 (0.46)
PFOS (mean (SD))	1.08 (0.56)	1.25 (0.58)	0.91 (0.49)	1.21 (0.8)	1.05 (0.91)	1.41 (1.71)
PFHxS (mean (SD))				1.09 (0.69)	0.99 (0.61)	1.22 (0.77)
Education (N (%))						
Low	99.0 (16.9)	42 (15.1)	57 (18.5)	46 (2.3)	38 (3.4)	8 (0.9)
Middle	391 (66.7)	173 (62.2)	218 (70.8)	871 (44.4)	542 (49.1)	329 (38.3)
High	96 (16.4)	63 (22.7)	33 (10.7)	1045 (53.3)	524 (47.5)	521 (60.7)
BMI (kg/m^2 (SD))	25.9 (4.0)	26.6 (3.5)	25.3 (4.4)	25.8 (4.5)	25.3 (4.7)	26.3 (4.2)
LDL (mmol/L (SD))	3.6 (0.9)	3.6 (0.9)	3.6 (1.0)	3.2 (0.9)	3.2 (0.9)	3.3 (0.,9)
HDL (mmol/L (SD))	1.6 (0.5)	1.3 (0.3)	1.8 (0.4)	1.7 (0.5)	1.9 (0.5)	1.4 (0.4)
Cholesterol (mmol/L (SD))	5.7 (1.1)	5.6 (1.0)	5.9 (1.1)	5.2 (1.0)	5.3 (1.0)	5.1 (1.0)
Triglycerides (mmol/L (SD))	1.2 (0.8)	1.4 (0.9)	1.0 (0.6)	1.3 (0.8)	1.1 (0.6)	1.5 (1.0)
Smoking (N (%))	66 (11.3)	31 (11.2)	35 (11.4)	269 (13.7)	134 (12.1)	135 (15.7)
Alcohol (g/day (SD))[‡]	14.0 (15.3)	18.9 (18.3)	9.6 (10.1)	19.4 (25.8)	15.4 (20.4)	24.7 (30.7)

Notes

[†] Number of missing values in the Rhineland Study: BMI (n= 13; 0.7%), LDL (n= 157; 8.0%), HDL (n= 157; 8.0%), Cholesterol (n= 157; 8.0%), Triglycerides (n= 157; 8.0%), Smoking (n= 0; 0%), Alcohol (n= 234; 11.9%)

[‡] In the Rhineland Study, alcohol intake of participants reporting an overall improbable caloric intake (<600 or >8,000) were excluded.

Abbreviations: Standard deviation (SD), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexanesulfonic acid (PFHxS), body mass index (BMI), low density lipoprotein (LDL), high density lipoprotein (HDL)

Table 2: Model estimates for the log-transformed clinical lipid measurement in the NEO study

Clinical Lipid Measurement	PFAS	Model 1†		Model 2‡				Age-stratified analysis±			
								Age ≤ 54		Age > 55	
		β *[95%CI]*	*p-value*	β *[95% CI]*	*p-value*	β interaction term *[95% CI]*	*p-value*	β *[95%CI]*	*p-value*	β *[95% CI]*	*p-value*
LDL	PFOA	0.018 [0.007; 0.033]	0.0412	0.292 [0.124; 0.461]	0.0007	-0.005 [-0.008; -0.002]	0.0014				
	PFOS	0.023 [0.004; 0.041]	0.0015	0.325 [0.165; 0.485]	**<0.0001***	-0.005 [-0.008; -0.003]	**0.0002***	0.044 [0.016; 0.071]	0.0020	0.007 [-0.016; 0.031]	0.538
HDL	PFOA	0.016 [0.004; 0.029]	0.0011	0.055 [-0.066; 0.175]	0.3716	-0.001 [-0.003; 0.001]	0.5279				
	PFOS	0.007 [-0.006; 0.021]	0.2624	-0.027 [-0.143; 0.088]	0.6405	0.001 [-0.001; 0.003]	0.5497				
Cholesterol	PFOA	0.02 [0.007; 0.033]	0.0025	0.235 [0.11; 0.359]	**0.0002***	-0.004 [-0.006; -0.002]	0.0007				
	PFOS	0.02 [0.007; 0.034]	0.0037	0.253 [0.134; 0.372]	**<0.0001***	-0.004 [-0.006; -0.002]	**0.0001***	0.036 [0.016; 0.057]	**0.0006***	0.009 [-0.009; 0.027]	0.3141
Triglycerides	PFOA	0.013 [-0.01; 0.036]	0.2780	0.182 [-0.041; 0.404]	0.1088	-0.003 [-0.007; 0.001]	0.1340				
	PFOS	0.016 [-0.009; 0.04]	0.2076	0.298 [0.087; 0.51]	0.0058	-0.005 [-0.009; -0.001]	0.0085				

Notes

†Model 1 was adjusted for sex, age and education

‡Model 2 was adjusted for sex, age and education and additionally included an age-interaction term

±The age-stratified analysis was only performed if the age-interaction term was significant and was adjusted for sex, age and education

***** Associations with the significant p-value after multiple test correction (p-value < 0.0007)

Abbreviations: Beta estimate (β), confidence interval (CI), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), low density lipoprotein (LDL), high density lipoprotein (HDL)

Table 3: Model estimates for the log-transformed clinical lipids in the Rhineland Study

Clinical Lipid Measurement	PFAS	Model 1†		Model 2‡				Age-stratified analysis≠			
								Age ≤ 54		Age > 55	
		β *[95%CI]*	*p-value*	β *[95% CI]*	*p-value*	β interaction term *[95% CI]*	*p-value*	β *[95%CI]*	*p-value*	β *[95% CI]*	*p-value*
LDL	PFOA	0.005 [-0.006; 0.016]	0.388	0.085 [0.038; 0.132]	**0.0004***	-0.001 [-0.002; -0.001]	**0.0005***	-0.006 [-0.024; 0.013]	0.551	0.008 [-0.005; 0.021]	0.2249
	PFOS	0.007 [-0.003; 0.017]	0.1726	0.166 [0.097; 0.235]	**<0.0001***	-0.002 [-0.003; -0.001]	**<0.0001***	0.016 [-0.019; 0.051]	0.3571	0.008 [-0.002; 0.018]	0.1127
	PFHxS	0.013 [0.003; 0.023]	0.0128	0.080 [0.037; 0.123]	**0.0002***	-0.001 [-0.002; -0.000]	0.0016				
HDL	PFOA	0.007 [-0.001; 0.016]	0.0690	0.001 [-0.034; 0.035]	0.9610	0.000 [-0.000; 0.001]	0.6956				
	PFOS	0.005 [-0.002; 0.013]	0.1575	0.002 [-0.049; 0.053]	0.9310	-0.000 [-0.001; 0.001]	0.9042				
	PFHxS	0.012 [0.004; 0.019]	0.0024	0.035 [0.003; 0.067]	0.0302	-0.000 [-0.001; 0.000]	0.1347				
Cholesterol	PFOA	0.008 [-0.001; 0.016]	0.0665	0.056 [0.020; 0.092]	0.0021	-0.001 [-0.001; -0.000]	0.0066				
	PFOS	0.007 [-0.001; 0.014]	0.0837	0.117 [0.064; 0.169]	**<0.0001***	-0.002 [-0.002; -0.001]	**<0.0001***	0.011 [-0.016; 0.037]	0.4415	0.008 [0.000; 0.016]	0.0403
	PFHxS	0.012 [0.004; 0.020]	0.0023	0.079 [0.046; 0.112]	**<0.0001***	-0.001 [-0.002; -0.001]	**<0.0001***	0.008 [-0.005; 0.021]	0.2539	0.005 [-0.004; 0.014]	0.2956
Triglycerides	PFOA	-0.001 [-0.015; 0.014]	0.9362	0.035 [-0.025; 0.095]	0.2590	-0.001 [-0.002; 0.000]	0.2376				
	PFOS	-0.005 [-0.018; 0.008]	0.4502	0.025 [-0.063; 0.114]	0.5830	-0.000 [-0.002; 0.001]	0.5054				
	PFHxS	-0.006 [-0.019; 0.007]	0.3969	0.048 [-0.008; 0.103]	0.0906	-0.001 [-0.002; 0.000]	0.0517				

Notes

†Model 1 was adjusted for sex, age and education

‡Model 2 was adjusted for sex, age and education and additionally included an age-interaction term

≠The age-stratified analysis was only performed if the age-interaction term was significant and was adjusted for sex, age and education

***** Associations with the significant p-value after multiple test correction (p-value < 0.0007)

Abbreviations: Beta estimate (β), confidence interval (CI), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexanesulfonic acid (PFHxS), low density lipoprotein (LDL), high density lipoprotein (HDL)

Table 4: Number of significant associations (p-value < 0.0007) per metabolite category with each PFAS in the NEO study. The range of the effect estimates are contained within the brackets.

NEO

	Model 1: Outcome ~ PFAS + Age + Sex + Education		Model 2: model 1 + PFAS*age		
	Number of positive associations (min estimate; max estimate)	Number of negative associations (min estimate; max estimate)	Number of positive associations (min estimate; max estimate)	Number of negative associations (min estimate; max estimate)	Number of age interactions (min estimate; max estimate)
PFOA					
Clinical lipid measures (n=4)	-	-	-	-	-
Amino acids (n=3)	-	-	-	-	-
Aromatic amino acids (n=2)	-	-	-	-	-
Branched-chain amino acids (n=3)	-	-	-	-	-
Apolipoproteins (n=3)	-	-	-	-	-
Cholesterol (n=9)	-	-	1 (1.410; 1.410)	-	1 (-0.023; -0.023)
Fatty acids (n=16)	-	-	-	-	-
Fluid balance (n=2)	-	-	-	-	-
Glycerides and phospholipids (n=9)	-	-	-	-	-
Glycolysis related metabolites (n=3)	-	-	-	-	-
Inflammation (n=1)	-	-	-	-	-
Ketone bodies (n=2)	-	-	-	-	-
Lipoprotein concentrations (n=14)	1 (0.150; 0.150)	-	-	-	-
Lipoprotein particle size (n=3)	-	-	-	-	-
HDL composition (n=44)	2 (0.145; 0.150)	-	-	-	-
LDL composition (n=33)	-	-	4 (1.403; 1.500)	-	4 (-0.024; -0.025)
VLDL composition (n=66)	-	-	-	-	-
IDL composition (n=11)	-	-	-	-	-
PFOS					
Clinical lipid measures (n=4)	-	-	2 (1.518; 1.575)	-	2 (-0.025; -0.025)
Amino acids (n=3)	-	-	-	-	-
Aromatic amino acids (n=2)	-	-	-	-	-
Branched-chain amino acids (n=3)	-	-	-	-	-
Apolipoproteins (n=3)	-	-	2 (1.392; 1.702)	-	2 (-0.028; -0.023)
Cholesterol (n=9)	-	-	5 (1.412; 1.634)	-	5(-0.027; -0.024)
Fatty acids (n=16)	3 (0.157; 0.185)	-	3 (1.507; 1.663)	-	3 (-0.026; -0.028)
Fluid balance (n=2)	-	-	-	-	-
Glycerides and phospholipids (n=9)	-	-	-	-	-
Glycolysis related metabolites (n=3)	-	-	-	-	-
Inflammation (n=1)	-	-	-	-	-
Ketone bodies (n=2)	-	-	-	-	-
Lipoprotein concentrations (n=14)	-	-	5 (1.408; 1.552)	-	5 (-0.026; -0.023)
Lipoprotein particle size (n=3)	-	-	-	-	-
HDL composition (n=44)	-	-	-	-	-
LDL composition (n=33)	-	-	20 (1.504; 1.903)	5 (-1.774; -1.456)	25 (-0.031; 0.029)
VLDL composition (n=66)	-	-	7 (1.298; 1.504)	-	7 (-0.025; -0.022)
IDL composition (n=11)	-	-	5 (1.407; 1.527)	1 (-1.313)	6 (-0.025; 0.023)

Table 5: Number of significant associations (p-value < 0.0007) per metabolite category with each PFAS in the Rhineland study. The range of the effect estimates are contained within the brackets.

Rhineland Study

	Model 1: Outcome ~ PFAS + Age + Sex + Education		Model 2: model 1 + PFAS*age		
	Number of positive associations N (min estimate; max estimate)	*Number of negative associations N (min estimate; max estimate)*	*Number of positive associations N (min estimate; max estimate)*	*Number of negative associations N (min estimate; max estimate)*	*Number of age interactions N (min estimate; max estimate)*
PFOA					
Clinical lipid measures (n=4)	-	-	1 (0.402; 0.402)	-	1 (-0.006; -0.006)
Amino acids (n=3)	-	-	-	-	-
Aromatic amino acids (n=2)	-	-	-	-	-
Branched-chain amino acids (n=3)	-	-	-	-	-
Apolipoproteins (n=3)	-	-	-	-	-
Cholesterol (n=9)	1 (0.080; 0.080)	-	-	-	-
Fatty acids (n=16)	-	-	-	-	-
Fluid balance (n=2)	1 (0.135; 0.135)	-	-	-	-
Glycerides and phospholipids (n=9)	1 (0.081; 0.081)	-	-	-	-
Glycolysis related metabolites (n=3)	-	-	-	-	-
Inflammation (n=1)	-	-	-	-	-
Ketone bodies (n=2)	-	-	-	-	-
Lipoprotein concentrations (n=14)	-	-	-	-	-
Lipoprotein particle size (n=3)	-	-	-	1 (-0.382; -0.382)	1 (0.006; 0.006)
HDL composition (n=44)	-	-	-	-	-
LDL composition (n=33)	-	-	-	-	-
VLDL composition (n=66)	-	-	-	-	-
IDL composition (n=11)	-	-	-	-	-
PFOS					
Clinical lipid measures (n=4)	-	-	2 (0.708; 0.781)	-	2 (-0.010; -0.009)
Amino acids (n=3)	-	-	-	-	-
Aromatic amino acids (n=2)	-	-	-	-	-
Branched-chain amino acids (n=3)	-	-	1 (0.761; 0.761)	-	1 (-0.010; -0.010)
Apolipoproteins (n=3)	-	-	1 (0.700; 0.700)	-	1 (-0.002; -0.002)
Cholesterol (n=9)	-	-	6 (0.573; 0.856)	-	6 (-0.011; -0.007)
Fatty acids (n=16)	-	-	9 (0.563; 1.025)	-	9 (-0.013; -0.008)
Fluid balance (n=2)	-	-	1 (0.620; 0.620)	-	1 (-0.009; -0.009)
Glycerides and phospholipids (n=9)	-	-	4 (0.558; 0.725)	-	4 (-0.009; -0.007)
Glycolysis related metabolites (n=3)	-	-	-	-	-
Inflammation (n=1)	-	-	-	-	-

Ketone bodies (n=2)	-	-	-	-	-
Lipoprotein concentrations (n=14)	-	-	5 (0.688; 0.838)	-	5 (-0.011; -0.009)
Lipoprotein particle size (n=3)	-	-	-	-	-
HDL composition (n=44)	-	-	2 (0.568; 0.580)	1 (-0.542)	3 (-0.008; 0.008)
LDL composition (n=33)	-	-	21 (0.637; 0.860)	7 (-0.603; -0.759)	28 (-0.011; 0.010)
VLDL composition (n=66)	-	-	12 (0.577; 0.808)	2 (-0.674; -0.716)	14 (-0.010; 0.009)
IDL composition (n=11)	-	-	7 (0.619; 0.847)	1 (-0.616; -0.616)	8 (-0.011; 0.008)
PFHxS					
Clinical lipid measures (n=4)	-	-	1 (0,480; 0.480)	-	1 (-0.007; -0.007)
Amino acids (n=3)	-	-	-	-	-
Aromatic amino acids (n=2)	-	1 (-0.105; -0.105)	-	-	-
Branched-chain amino acids (n=3)	-	-	-	-	-
Apolipoproteins (n=3)	1 (0.088; 0.088)	-	-	-	-
Cholesterol (n=9)	6 (0.073; 0.094)	-	3 (0.372; 0.416)	-	3 (-0.006; -0.005)
Fatty acids (n=16)	9 (0.078; 0.095)	-	3 (0.336; 0.401)	-	3 (-0.006; -0.005)
Fluid balance (n=2)	1 (0.092; 0.092)	-	-	-	-
Glycerides and phospholipids (n=9)	2 (0.077; 0.078)	-	2 (0.397; 0.410)	-	2 (-0.006; -0.006)
Glycolysis related metabolites (n=3)	-	-	-	-	-
Inflammation (n=1)	-	-	-	-	-
Ketone bodies (n=2)	-	-	-	-	-
Lipoprotein concentrations (n=14)	2 (0.081; 0.086)	-	-	-	-
Lipoprotein particle size (n=3)	-	-	-	-	-
HDL composition (n=44)	1 (0.076; 0.076)	-	-	-	-
LDL composition (n=33)	11 (0.081; 0.089)	-	-	-	-
VLDL composition (n=66)	4 (0.076; 0.103)	-	-	-	-
IDL composition (n=11)	2 (0.082; 0.089)	-	-	-	-