From Institute of Environmental Medicine
Karolinska Institutet, Stockholm, Sweden

PERSISTENT ENVIRONMENTAL POLLUTANTS AND RISK OF CARDIOVASCULAR DISEASE

Tessa Schillemans

Karolinska Institutet

Stockholm 2023

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8016-932-5

Persistent Environmental Pollutants and Risk of Cardiovascular Disease

Thesis for Doctoral Degree (Ph.D.)

By

Tessa Schillemans

The thesis will be defended in public at Karolinska Institutet in the Rockefeller room, Nobels väg 11, Solna, Sweden at 9:00 on 24 March 2023.

Principal Supervisor:
Professor Agneta Åkesson
Karolinska Institutet
Institute of Environmental Medicine
Unit of Cardiovascular and Nutritional Epidemiology

Co-supervisor(s):
Associate Professor Carl Brunius
Chalmers University of Technology
Department of Life Sciences
Division of Food and Nutrition Sciences

Associate Professor Karin Leander
Karolinska Institutet
Institute of Environmental Medicine
Unit of Cardiovascular and Nutritional Epidemiology

Dr. Carolina Donat-Vargas
ISGlobal, Barcelona
Karolinska Institutet

Opponent:
Associate Professor Tony Fletcher
London School of Hygiene and Tropical Medicine
Department of Public Health, Environments & Society

Examination Board:
Associate Professor Eva Andersson
University of Gothenburg
Department of Occupational and Environmental Medicine

Professor Jonathan Martin
Stockholm University
Department of Environmental Science and SciLifeLab

Professor, MD, Gunnar Engström
Lund University
Department of Clinical Sciences

To my parents.

Popular science summary of the thesis

Humans are exposed to a plethora of synthetic chemicals, which potentially interact with normal human physiology. The focus of this thesis was on per- and polyfluoroalkyl substances (PFAS), a group of over 4,700 man-made chemicals. PFAS can be detected almost everywhere on our planet as well as in humans and received the nickname 'forever chemicals', as they are virtually impossible to break down. PFAS became notorious when high contaminations shook up society. Ever since, PFAS effects on human health have been a topic of investigation in toxicology and epidemiology.

We performed several observational studies to investigate whether exposure to PFAS impacted obesity, type 2 diabetes, myocardial infarction and stroke, which are some of the major societal challenges of the twenty-first century. We furthermore investigated small molecules in the blood that correlated with PFAS exposure to gain more mechanistic insight in how PFAS may impact human physiology. Our studies indicated that exposure to PFAS correlates with several intermediate products of metabolic reactions in the human body, which indicates there may be PFAS-induced disturbances in metabolism. We furthermore found evidence for associations of PFAS exposure with cardiometabolic diseases and their risk factors: lower BMI in teenagers (although not for all PFAS), metabolic patterns linked to both lower and higher risk of type 2 diabetes in adults, higher cholesterol levels, lower triglyceride levels and lower risk of myocardial infarction.

These studies showed no associations of PFAS with increased disease risk, at exposure levels typically found in the general population. Although this is a positive finding for human health, this does not mean that PFAS is harmless. We found indications for PFAS interactions with human physiology through metabolic regulation. Since all processes in the human body are interconnected, there may be risk for unforeseen and undesirable outcomes. In addition, our findings illustrate the complexity and multitude of exposures humans face. When we investigated small molecules in the blood that correlated with PFAS exposure, we also found other synthetic chemicals and food-related intermediates. All these different exposures can impact biological mechanisms, potentially in different manners, which makes it difficult to disentangle individual compound effects. For example, we included other persistent pollutants in our analyses and found that they had opposite associations with risk myocardial infarction and stroke as compared to PFAS. Even within the PFAS chemical group, we have shown that PFAS compounds can associate differently with health outcomes and that PFAS may affect multiple metabolic pathways which could have different effects on disease risk.

This thesis underscores the complexity of the relationship between exposures and health. The results indicate that there is an effect of man-made, widespread, persistent organic pollutants on human physiology. This is important knowledge as we work towards improving planetary health and building a more sustainable future.

Abstract

Persistent chemicals emitted in the environment can have a considerable impact on ecosystems and human health, now and in the future. One notorious group of persistent organic pollutants (POPs) is the per- and polyfluoroalkyl substances (PFAS). Since their production in 1940s for household and consumer products, they have accumulated in the environment and in humans via consumption of contaminated drinking water and food. They are hypothesized to induce metabolic disturbances, due to shared chemical similarities with fatty acids. Consequently, PFAS may have high societal and economic impact by increasing risk of obesity, type 2 diabetes (T2D) and cardiovascular disease (CVD). However, reports on these associations are scarce, and the underlying molecular pathways are still unclear. Therefore, in this PhD project, we aimed to **i)** investigate associations between PFAS and risk of several cardiometabolic diseases and **ii)** explore potential underlying pathways.

In **Paper I**, we investigated cross-sectional associations between PFAS mixtures and body mass index (BMI) in European teenagers using meta-regression. Results showed a tendency for inverse associations between PFAS and BMI and indicated a potential for diverging contributions between PFAS compounds. In **Paper II**, using a nested case-control study on T2D including metabolomics data in Swedish adults, we found that PFAS correlated positively with glycerophospholipids and diacylglycerols. But whilst glycerophospholipids associated with lower T2D risk, diacylglycerols associated with higher T2D risk. This indicates a potential for diverging effects on disease risk. In **Paper III**, we investigated whether genetic polymorphisms in *peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PPARGC1A)*, which encodes a master regulator of pathways potentially disrupted by PFAS exposure, associated with secondary cardiovascular events in a large consortium study. However, we did not find clear evidence for such associations. In **Paper IV**, we assessed associations of PFAS with blood lipids and incident CVD using case-control studies nested in two Swedish adult cohorts. We observed overall null associations with stroke, but a tendency for inverse associations with myocardial infarction as well as associations with higher HDL-cholesterol and lower triglycerides, but also with higher LDL-cholesterol. In **Paper V**, we included OMICs data (metabolites, proteins and genes), which linked PFAS to lower myocardial infarction risk via lipid and inflammatory pathways. Likewise, a group of 'old POPs', the organochlorine compounds (OCs), were linked to higher myocardial infarction risk via the same pathways and to higher stroke risk via mitochondrial pathways.

Thus, although we found no evidence for associations between PFAS and increased cardiometabolic disease risk, the overall findings indicate associations of PFAS with metabolic disturbances, particularly lipid metabolism. This is a potential adverse effect on human physiology and warrants further attention.

List of scientific papers

I. **Schillemans T**, Iszatt N, Remy S, Schoeters G, Fernández MF, D'Cruz SC, […], Åkesson A. Cross-sectional associations between exposure to per- and polyfluoroalkyl substances and body mass index among European teenagers in the HBM4EU aligned studies. *Environmental Pollution*. 2022 Nov;316(Pt 1):120566.

II. **Schillemans T**, Shi L, Donat-Vargas C, Hanhineva K, Tornevi A, Johansson I, Koponen J, Kiviranta H, Rolandsson O, Bergdahl IA, Landberg R, Åkesson A, Brunius C. Plasma metabolites associated with exposure to perfluoroalkyl substances and risk of type 2 diabetes – A nested case-control study. *Environment International*. 2021 Jan;146:106180.

III. **Schillemans T**, Tragante V, Maitusong B, Gigante B, Cresci S, Laguzzi F, […], Leander K. Associations of polymorphisms in the peroxisome proliferator-activated receptor gamma coactivator-1 alpha gene with subsequent coronary heart disease: an individual-level meta-analysis. *Frontiers of Physiology*. 2022 Jun 23;13:909870.

IV. **Schillemans T**, Donat-Vargas C, Lindh CH, de Faire U, Wolk A, Leander K, Åkesson A. Per- and Polyfluoroalkyl Substances and Risk of Myocardial Infarction and Stroke: A Nested Case–Control Study in Sweden. *Environmental Health Perspectives*. 2022 Mar;130(3):37007.

V. **Schillemans T**, Yan Y, Ribbenstedt A, Donat-Vargas C, Lindh CH, Kivirante H, Wolk A, Landberg R, Åkesson A, Brunius C. OMICs signatures linking persistent organic pollutants to cardiovascular disease in the Swedish Mammography Cohort. *Manuscript*.

Scientific papers not included in the thesis

Schillemans T*, Shi L*, Liu X, Åkesson A, Landberg R, Brunius C. Visualization and Interpretation of Multivariate Associations with Disease Risk Markers and Disease Risk-The Triplot. *Metabolites*. 2019 Jul;9(7):133.

Schillemans T, Bergdahl IA, Hanhineva K, Shi L, Donat-Vargas C, Koponen J, Kiviranta H, Landberg R, Åkesson A, Brunius C. Associations of PFAS-related plasma metabolites with cholesterol and triglyceride concentrations. *Environmental Research*. 2022 Oct;216(Pt2):114570.

Yuan S, Titova OE, Zhang K, Gou W, **Schillemans T**, Natarajan P, Chen J, Li X, Åkesson A, Bruzelius M, Klarin D, Damrauer SM, Larsson SC. Plasma protein and venous thromboembolism: prospective cohort and mendelian randomisation analyses. *British Journal of Haematology*. 2023 Feb; 00: 1– 10.

* Authors contributed equally.

Contents

List of abbreviations

60YO	The cohort of 60-year-olds
ApoB	Apolipoprotein B
ApoA1	Apolipoprotein A1
BMI	Body mass index
CVD	Cardiovascular disease
DG	Diacylglycerols
GENIUS-CHD	Genetics of subsequent coronary heart disease
GPL	Glycerophospholipids
HBM4EU	Human biomonitoring for Europe
HDL	High-density lipoprotein
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
NR	Nuclear receptor
OC	Organochlorine compound
PCA	Principal component analysis
PFAS	Per- and polyfluoroalkyl substances
PFDA	Perfluorodecanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFUnDA	Perfluoroundecanoic acid
POP	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptor
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
SMC	Swedish mammography cohort
SMC-C	Swedish mammography cohort-clinical
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
VIP	Västerbotten Intervention Programme

1 Introduction

Planetary health is one of the great challenges for the 21st century. It refers to analyzing and addressing the impacts of human disruptions to Earth's natural systems on human health and all life on Earth [1]. The concept recognizes that the health of human civilization depends on the state of the natural systems. Within this field, the pollution of the Earth by human inventions plays a large part. Chemical persistent contaminants, created and emitted by humans, may disrupt ecosystems upon which we depend and/or directly affect human health as they end up in human food chains. One group of contaminants, called per- and polyfluoroalkyl substances (PFAS), are now broadly found in the environment, sometimes in extremely high amounts, where they may unsettle ecosystems and wildlife. Furthermore, they are found in drinking water and food, potentially also directly impacting human health.

PFAS have structural resemblance to fatty acids and are suggested to disrupt lipid metabolism, with potential consequences for cardiometabolic health. As cardiometabolic diseases together by far outweigh all other causes of death globally, knowledge on preventable risk factors is imperative for population health. Therefore, the effect of PFAS on metabolic processes and its consequent impact on cardiometabolic diseases needs to be clarified. The field of epidemiology aims to study the distribution and determinants of health-related states and events in populations. Within this field, environmental exposures are investigated in relation to health and disease.

The focus of this thesis is to provide stronger epidemiological evidence on potential metabolic perturbations as well as risk of CVD resulting from PFAS exposure and to use novel OMICs techniques to gain insight in the molecular pathways involved. The results are important for risk assessments and to, if necessary, take preventive actions to control PFAS manufacture and use to improve planetary health.

2 Background

2.1 Cardiovascular and other cardiometabolic diseases

2.1.1 Cardiovascular disease

The term cardiovascular disease (CVD) includes disorders of the heart and blood vessels. This class of diseases is the leading cause of mortality globally, accounting for approximately 20.5 million deaths each year [2,3]. In survivors, they may result in severe permanent disability. The highest number of CVD cases are found in low- and middle-income countries [4]. The most commonly occurring CVD deaths relate to ischemic heart disease, such as myocardial infarction, or to stroke [5]. Myocardial infarction occurs due to reduced blood flow to a part of the heart, whereas for stroke the blood flow to the brain is cut off. Myocardial infarction has a relatively uniform etiology and is in most cases caused by atherosclerotic plaque rupture with superimposed *in-situ* arterial thrombosis [6]. In contrast, ischemic stroke has multiple possible causes ranging from plaque rupture with *in-situ* thrombosis and cardioembolic, arterioembolic, lacunar (small-vessel occlusion) or unknown causes [7]. Ischemic stroke is the most common form of stroke, the remaining one-third is represented by hemorrhage [8].

CVD is a multifactorial disease with risk factors that include sex, age, family history, ethnicity and socioeconomic status as well as other modifiable risk factors such as, smoking, unhealthy diet, low physical activity, high alcohol consumption and other environmental toxicants (*e.g.* air pollution) [9]. In addition, dyslipidemia, abdominal obesity, hypertension and type 2 diabetes (T2D) increase CVD risk [10]. CVD has an important genetic component as well as potential gene-environment interactions. CVD is considered polygenic, with many variants in different genes accounting for small increases in risk and some of them relate to blood lipid levels or blood vessel reactivity [11]. CVD can be prevented by lifestyle changes such as healthy diet and physical activity as well as treatment with drugs, which often exert their effect through lowering the blood lipid levels or blood pressure [12]. It is also increasingly described as a chronic disease with a dynamic nature and due to increased survival rates after a first-time event, many patients are living with CVD. Still only little is known about the risk factors influencing its progression after the first event.

Symptoms of myocardial infarction include pressure or pain in the chest, shortness of breath, anxiety, nausea and vomiting. Stroke is characterized by sudden numbness in the face or limbs on one side of the body, confusion or speech impediment, loss of sight or coordination and headache. Fast treatment is imperative for survival.

2.1.2 Physiopathology of cardiovascular disease

Lipids are surrounded by phospholipids, free cholesterol and apolipoproteins during their transport through the body. There are different classes of lipid particles, called lipoproteins, that differ in function, size, lipid composition and apolipoproteins. The apolipoproteins aid in formation of lipoproteins and act as ligands for receptors or as activators or inhibitors of enzymes involved in lipoprotein metabolism [13]. **Figure 1** illustrates the transport of cholesterol from intestine to liver to peripheral tissues and its reverse transport back to the liver and intestine [13].

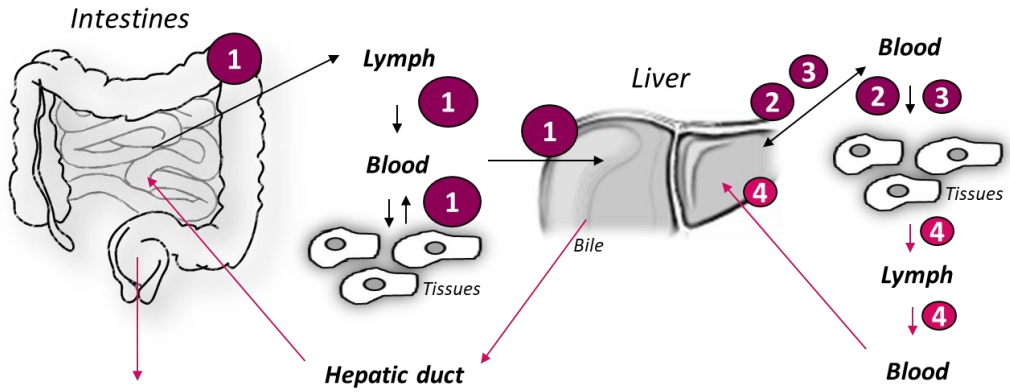


Figure 1. A schematic overview of lipid metabolism in humans. Lipid particles are indicated as 1) Chylomicron 2) VLDL 3) LDL 4) HDL. (Source: Personal Collection)

1) Dietary cholesterol and triglycerides taken up in the intestine are packaged into chylomicrons and delivered to the liver via the lymphatic system and bloodstream. On the way, triglycerides from the chylomicrons are metabolized by lipoprotein lipase and the released free fatty acids are taken up by muscle or adipose tissue for energy or storage. Chylomicron-remnants are then taken up in the liver via Low-Density Lipoprotein (LDL)-receptors (LDL-R).

2) In the liver, remnants of dietary and endogenous cholesterol (synthesized from fatty acids via several enzymatic reactions, the rate-limiting being catalyzed by HMG-CoA reductase; HMGCR) together with triglycerides are packaged in Very Low-Density Lipoproteins (VLDL). This carries triglycerides from the liver to tissues via the blood, which turns it into Intermediate-Density Lipoproteins (IDL).

3) Intermediate-Density Lipoprotein is further converted to LDL, which is taken up by the LDL-receptor and delivers cholesterol to tissues (including liver) via the bloodstream.

4) The reverse cholesterol transport chain starts with synthesis of apolipoproteins, which bind cholesterol and phospholipids in the liver and intestine to form High-Density Lipoproteins (HDL). HDL then acquires excess cholesterol from other tissues and lipoproteins in the bloodstream to transport it back to the liver via lymph. In the liver, cholesterol can be converted into bile acids (catalyzed by cholesterol 7-alpha hydroxylase; CYP7A1) for the intestine, where it may be re-absorbed or excreted.

Lipid metabolism and atherosclerosis are closely connected. Pro-atherogenic lipoproteins circulating in the blood (*e.g.* chylomicron-remnants, intermediate-density lipoprotein and especially LDL) can induce plaque formation [14], as illustrated in **Figure 2**.

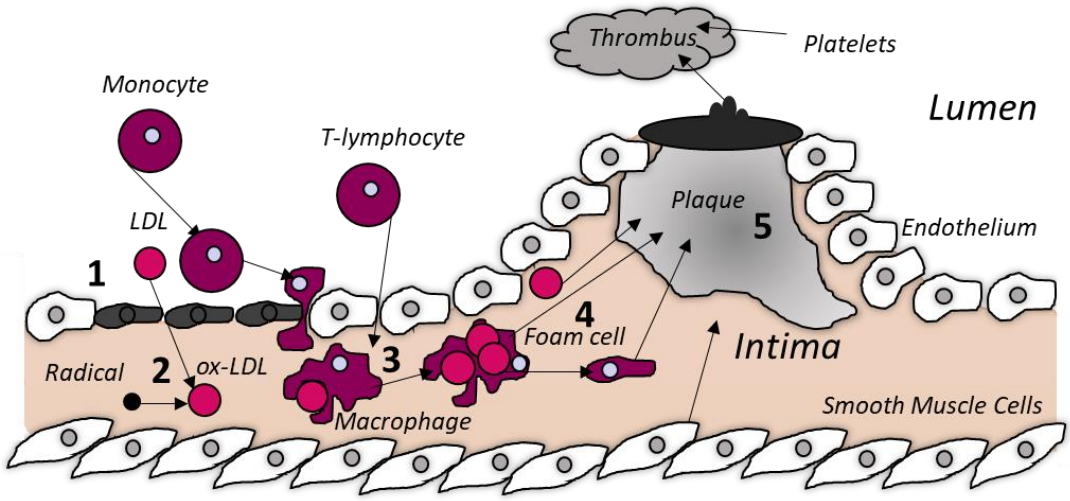


Figure 2. A schematic overview of the five key steps in atherogenesis: 1) endothelial dysfunction, 2) lipids in the intima, 3) migration of leukocytes and smooth muscle cells, 4) foam cell formation and 5) degradation of extracellular matrix and plaque formation. (Source: Personal Collection).

1) Endothelial dysfunction can be caused by hemodynamic (*i.e.* high blood pressure) or chemical stressors (*e.g.* smoking).

2) When this happens, lipoproteins can pass into the intima and bind to proteoglycans leading to subendothelial accumulation of lipoproteins. They then can become glycated or oxidized by free radicals.

3) This elicits an innate immune response, causing migration of leukocytes into the vessel wall. They become macrophages and engulf oxidized lipoproteins. In more advanced lesions, smooth muscle cells can also transmigrate to the intima.

4) The engulfment of excessive oxidized lipoproteins transforms the macrophages into foam cells. The foam cells are unable to migrate out of the intima and eventually die, which creates the necrotic core of the plaque.

5) The extracellular matrix plays a role in plaque integrity and stability. Acceleration of inflammation and apoptosis leads to thinning of the fibrous cap enclosing the necrotic core of the plaque, making rupture more likely. After rupture, platelets play a role in thrombus formation resulting in clinical events.

Steps 1 and 2 present the early stage of atherosclerosis, the fatty streak, which is followed by plaque progression in steps 3, 4 and 5. The last stage, the clinical complication of atherosclerosis, presents itself if the plaque ruptures in step 5, which may induce *e.g.* myocardial infarction or ischemic stroke [14].

2.1.3 Type 2 diabetes

T2D is a chronic disease where tissues become resistant to insulin, reducing the uptake of glucose into the cells and leading to high blood glucose levels. Over time, this can induce damage to the organs and tissues. Prevalence of T2D has increased over the last decades, particularly in low- to middle-income countries where it has great socio-economical costs. Approximately 380 million people worldwide are living with T2D [15].

T2D is also a multifactorial disease where genetic and environmental factors interact. Risk factors for T2D include age, obesity, family history, ethnicity and co-morbidities such as history of CVD, dyslipidemia and hypertension [15]. Some risk factors can be used as intermediate markers such as high cholesterol or triglyceride levels. T2D also gives an increased risk for all-cause mortality and CVD [15]. Besides genes, lifestyle-related factors play an important role in T2D development, particularly diet, exercise, smoking and alcohol consumption [15]. Likewise, environmental toxicants, some of them acting as endocrine-disrupting chemicals, have emerged as novel diabetes risk factors [16].

The etiology and pathogenesis of T2D is a complex interplay between genes and environment which results in disturbed metabolic processes, although the specific mechanisms involved are not fully understood. However, in general, several steps can be recognized: First, insulin resistance develops in the tissues through disruption of the insulin signals potentially through genetic factors in insulin receptors or substrates, as well as via inflammatory mediators and free fatty acids or triglycerides [17]. Then, pancreatic β cells try to compensate the insulin resistance in the tissues by producing more insulin, which eventually leads to apoptosis of the β cells and decreased insulin secretion [17]. As the disease progresses the high blood glucose levels may then cause damage to the blood vessels and organs leading to clinical complications.

2.1.4 Obesity

T2D and CVD share several risk factors and obesity is an important one. Obesity is excessive fat accumulation posing risks to health. It is often measured as body mass index (BMI; weight/height^2) where having a BMI over 25 and 30 is classified as overweight and obese, respectively, or as waist circumference. It is also on the rise in low- and middle-income countries and currently there are more people obese than underweight [18]. It is especially related to unhealthy diet and low physical activity, but there may be other risk factors such as endocrine disrupting chemicals (particularly during the early years of development) [19]. Obesity impacts life quality as well as increases the risk of multiple chronic diseases and this starts already during childhood [20,21].

2.2 Per- and polyfluoroalkyl substances

2.2.1 History and background

One group of environmental pollutants that has been suggested to impact cardiometabolic diseases is the per- and polyfluoroalkyl substances (PFAS). PFAS have been produced since the 1940s by a variety of industries around the globe for a diverse array of household and consumer products. As a result of this widespread use and due to their high persistence, they are found almost ubiquitously in the environment, animals and humans [22,23]. Ground and surface waters surrounding airports and military bases using fire-fighting foam during training activities as well as industrial sites that produce PFAS or PFAS-products can contain very high PFAS concentrations [24,25], which has led to severe consequences for the environment, wildlife and human health in the past. Perhaps the most well-known is the contamination from a DuPont factory in Mid-Ohio (West Virginia, US), instigating a two-decade long lawsuit and a scientific panel [26]. A similar situation occurred in Ronneby (Sweden) where drinking water had been contaminated via fire-fighting foam from military training during 1980 to 2013 [25,27].

Currently, over 4700 different per- and polyfluorinated compounds have been identified [28]. PFAS commonly consist of a carbon backbone with at least one perfluorinated methyl or methylene group [29]. There are different functional groups, *e.g.* carboxylic (R-COOH) and sulfonic (R-SO3H) (**Figure 3**), or chain lengths, *e.g.* short- and long-chain [30]. Carboxylic PFAS with more than six or sulfonic PFAS with more than 5 perfluorinated carbons are defined as long-chain PFAS [30]. PFAS production using electrochemical fluorination results in linear and branched isomers, whilst a relatively newer approach of telomerization results in pure linear product [31]. Their chemical structure makes them both hydrophobic and lipophobic, thus they are stain- and water- repellent [30]. Therefore, they are useful in products that need to be able to withstand water or grease such as coatings for furniture, cooking ware, clothes and shoes as well as food packaging or fire-fighting foam.

2.2.2 Environment and human exposure

PFAS can partition in both solid and aquatic materials and due to their high stability in solutions they accumulate in water and marine mammals [32]. The long-chain or sulfonic PFAS seem to be more bio-accumulative than their short-chain or carboxylic counterparts [33], while on the other hand, short-chain PFAS have higher mobility [34]. PFAS can end up in the environment via production or waste streams travelling through air or water, leading to contaminated ground and drinking water (**Figure 3**). It is estimated that drinking water can contribute up to 75% of human exposure in contaminated areas and up to 86% of dietary exposure relates to fish and seafoods [35,36]. Furthermore, wastewater treatment plants do not effectively remove PFAS, leading to increased PFAS concentrations in the reclaimed wastewater or biosolids used for land application [32]. In this manner, PFAS could pollute water, soil, crops, livestock and wildlife, which creates

other dietary human exposure sources such as meat, eggs, dairy and crops. Other routes of human exposure are from PFAS-containing food packages, inhalation of house dust (to a lesser extent) and dermal exposure from products (minor) [36].

2.2.3 Accumulation in humans

PFAS are detected in human blood samples from all over the world [23], they are furthermore found in bone, lung, liver and kidney tissues [35,37]. PFAS are absorbed in the gastrointestinal tract, distribute through the body without being metabolized and accumulate in the liver [35,37]. Elimination routes are urine and bile and for women additionally breast milk and menstruation fluids, which may be the reason for lower PFAS levels in women compared to men [23]. However, due to high reabsorption in the kidneys and gastrointestinal tract, PFAS have a long half-life ranging from weeks (for short-chain) to several years (for long-chain) with estimations of 3.4 and 2.7 years for perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), respectively [38,39]. However, half-lives up to 5-8 years have also been reported [40]. Furthermore, the intraclass correlations based on repeated measurements in individuals approximately 10 years apart were estimated to be quite high (0.52-0.85) [41].

2.2.4 Risk assessment and regulations

International organizations (such as the International Agency for Research on Cancer (IARC)) and regulatory agencies in several countries or regions (such as the European Food Safety Agency (EFSA), the Agency for Toxic Substances and Disease Registry (ATSDR) and the US Environmental Protection Agency (EPA)) have evaluated the scientific literature on PFAS exposure and toxicology to identify and assess the potential for human health effects. Potential health effects include: increase in serum total cholesterol in adults, decrease in antibody response at vaccination in children, reduced birth weight, increased prevalence of high serum levels of the liver enzyme alanine aminotransferase (liver damage), obesity, cancer (kidney and testicular), thyroid hormone disruption, ulcerative colitis (inflammatory bowel disease), increased asthma diagnosis, endocrine disruption, decreased fertility, pregnancy induced hypertension/pre-eclampsia, growth, learning and development in children [35,36,42-44]. The current level of evidence is strongest for the first four (**Figure 3**), although the modes of action are still largely unknown [35,45].

Regulatory actions based on this knowledge include the establishment of a tolerable weekly intake in Europe by EFSA of 13 and 6 ng/kg body weight per week for PFOS and PFOA, respectively, based on increased serum cholesterol [35], which was recently updated to 4.4 ng/kg body weight per week for a sum of four prevalent PFAS based on decreased vaccination response [45]. The high level of global concern for PFAS uses and emissions is reflected by the dedication of a project and web-portal to PFAS by the Organisation for Economic Co-operation and Development (OECD) since 2015. Actions to phase out PFOS and PFOA were first taken by the Stockholm Convention in 2009 and 2015, respectively.

The complexity of regulating PFAS is illustrated by the decision to restrict some PFAS globally under the persistent organic pollutants (POPs) regulation (PFOS 2009, PFOA 2020, perfluorohexane sulfonic acid; PFHxS 2023), whilst other PFAS are regulated in Europe under the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation (covering around 200 C9-C14 perfluorinated carboxyl acids 2023) and some are listed as substances of very high concern [46]. Recently, a new proposal to the European chemical agency aims to restrict around 10,000 PFAS under REACH legislation, which would be one of the largest chemical substances bans ever in Europe.

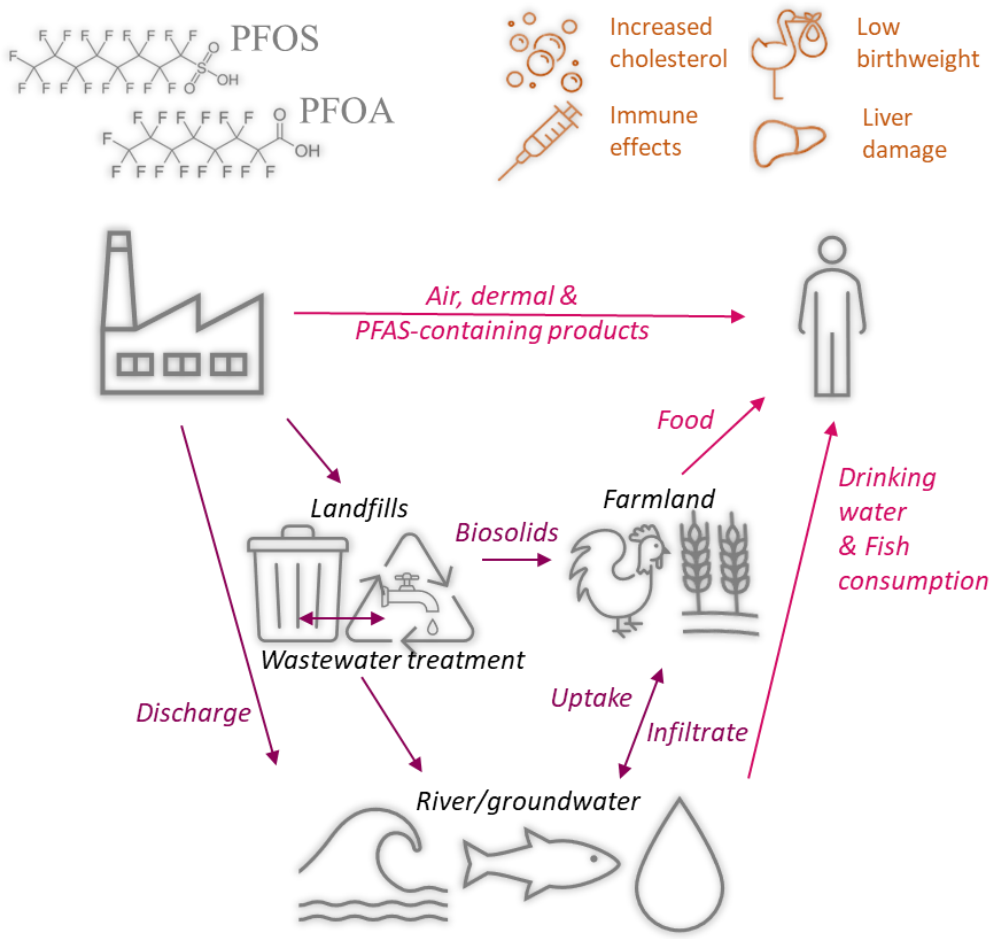


Figure 3. A schematic overview of human exposure to PFAS and adverse health effects. Abbreviations: FFF, fire-fighting foam; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; WWTP, wastewater treatment plant. (Source: Personal Collection)

2.2.5 Other persistent organic pollutants

This thesis emphasizes PFAS and human health, but PFAS is by no means the only environmental pollutant that humans are exposed to. PFAS are classified as POPs together with several other groups of synthetic chemicals, such as the organochlorine compounds (OCs) from 'old' industrial and consumer products (*e.g.* polychlorinated biphenyls, PCBs) or pesticides (*e.g.* dichlorodiphenyltrichloroethane, DDT). Several of these POPs share exposure pathways (*e.g.* fatty fish intake), may affect similar molecular pathways and have also been linked to cardiometabolic diseases [47].

2.3 Per- and polyfluoroalkyl substances and cardiometabolic disease

2.3.1 Molecular pathways

There are several hypotheses for pathways linking PFAS exposure to cardiometabolic perturbations. First, the liver is an important metabolic organ and mechanistic studies indicate disruptions in lipid metabolism and liver function upon PFAS exposure [48]. This seems to be induced via activation of nuclear receptors (NRs). NRs are transcription factors that normally respond to intracellular signals, hormones or dietary lipids, including receptors for oxysterols (LXRs), bile acids (CAR, FXR, PXR) and fatty acids (PPARα/γ/δ, HNF4α) [49]. They are important regulators of lipid metabolism including the absorption, synthesis and re-modelling in the liver, mobilization, reverse transport, bile acid synthesis and bile acid re-absorption [49–54]. As PFAS' chemical structure bears resemblance to fatty acids, they may induce metabolic changes via binding to these receptors [55–57]. This may impact obesity, T2D and CVD via elevation of lipoproteins in the circulation [35,58]. Two hypothesized pathways are illustrated in **Figure 4** relating to peroxisome proliferator-activated receptor α (PPARα) - triglycerides and hepatocyte nuclear factor 4α (HNF4α) – cholesterol [59,60].

Second, the immune system and inflammation are relevant for cardiometabolic diseases and are, in part, also regulated by NRs [61]. PFAS has been shown to associate with immunological and inflammatory processes [62,63]. On one hand, PFAS may have antioxidant and anti-inflammatory effects via activation of PPARα [61] or via upregulation of phosphatidylcholine synthesis [48]. On the other hand, PFAS has been shown to associate with oxidative stress and mitochondrial dysfunction [64]. Also, PFAS may cause endothelial dysfunction via reactive oxygen species induction [65,66], enhanced platelet aggregation via PFAS accumulation in platelet membranes which alters their configuration [67,68] and increasing the oxygen-carrying capacity of blood cells [69]. Lastly, disruptions of the gut microbiome, induced by chemicals such as PFAS, may impact both metabolism and immunity [70,71].

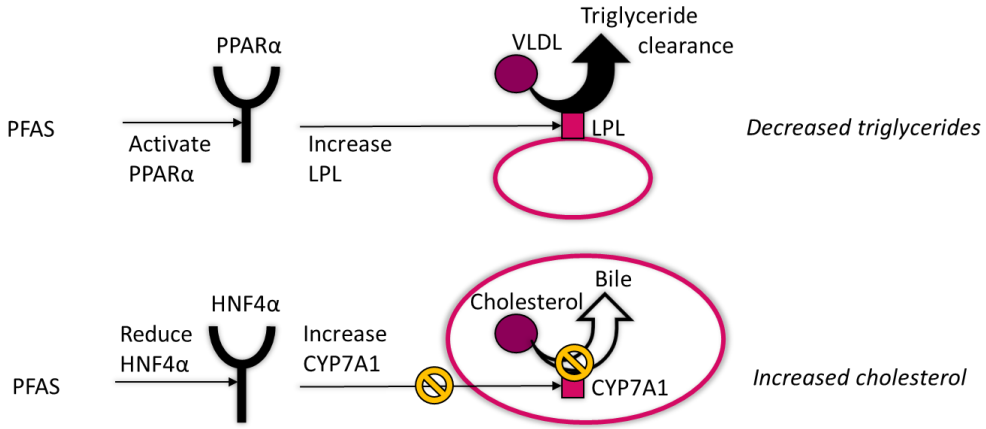


Figure 4. Schematic view of PFAS activating peroxisome proliferator-activated receptor α (PPARα), which increases lipoprotein lipase (LPL) expression and triglyceride clearance by LPL (top) and PFAS reducing hepatocyte nuclear factor 4α (HNF4α) expression, which reduces cholesterol 7 α-hydroxylase (CYP7A1) expression and cholesterol conversion to bile by CYP7A1 (bottom). (Source: Personal Collection)

2.3.2 PPAR gamma coactivator-1 alpha

Many of these NRs are coactivated by PPAR gamma coactivator-1 alpha (PPARGC1A). PPARGC1A is therefore termed a key modulator of energy metabolism [72] and has also been suggested as one of the potential mechanisms for cross-talks between NRs [54]. There are several pieces of evidence indicating that this regulator and its pathways are important for CVD. Genetic single nucleotide polymorphisms (SNPs) in the *PPARGC1A* gene are associated with 1) several processes important for atherosclerosis such as lipid homeostasis [72], endothelial function and inflammation [73] as well as 2) other risk factors such as adiposity, T2D [74], hypertension [75] and cholesterol levels [76] and additionally 3) first time CVD [77-79]. This strengthens the hypothesis that pathways under regulation of PPARGC1A may be involved in CVD and thus, that PFAS may impact CVD risk if it indeed affects NRs involved in these pathways.

2.3.3 Animal studies

In contrast to humans, animal studies investigating PFAS exposure and blood lipid levels generally show more of an inverse association between PFAS and cholesterol as well as triglycerides [35,80]. In addition, a more favorable effect of PFAS on T2D has also been shown [81]. However, one study indicated that animals fed on high-fat diets have increased cholesterol levels upon PFAS exposure and they hypothesized that diet or body composition may modify the effect of PFAS on the lipid metabolism [82]. In contrast, a study using mice with a humanized lipid metabolism system (APOE*3 Leiden mice) was unable to show such an effect with either normal or high-fat diet [83]. It is important to keep in mind the differences between laboratory animals and humans in lipid metabolism and CVD development [84] (*i.e.* cholesterol 7-alpha hydroxylase is responsible for 90% of bile acid production in humans, but only 60% in mice, cholesteryl ester transfer protein plays a

large role in transferring triglycerides and cholesteryl esters between lipoproteins in humans but is absent in mice and mice carry most cholesterol in HDL instead of LDL). There are also differences in PFAS dose and metabolism (*i.e.* the half-life may be years in humans, while hours or days in most laboratory animal species) [23] and in expression and potency of NRs (*i.e.* humans have lower expression of PPARα in the liver and require higher doses of PPARα activators to trigger its transcriptional activity compared to most laboratory animal species) [85,86]. Thus, caution is required to extrapolate results from animals to humans.

2.3.4 Human studies

As it is, the human evidence for associations between PFAS and increased cholesterol is considered quite strong [35,42] and several studies indicated direct associations [25,87-90], although one longitudinal study also showed null findings [91]. In addition, causality is not fully established as reverse causality could occur due to shared excretion mechanisms of PFAS and cholesterol in the bile [35,92], although a longitudinal study with an intervention on PFAS contaminated drinking water [89] and a study using exposure based on PFAS intake dispute that [25]. There are less reports on triglyceride levels and although animal studies seem to find mainly inverse associations, potentially due to accumulation of lipids in the liver, both direct [88] and inverse [91] associations are found in humans.

Interestingly, one study also found interactions between PFAS and BMI with more apparent blood lipid associations in obese persons. The authors hypothesized that liver steatosis, for which obese persons are more susceptible, may play a role in modifying the associations between PFAS and lipids [93]. This is supported by several animal studies [82,94,95] that show effects of PFAS on hepatic pathways and steatosis. Similarly, a human lifestyle intervention study found that obesogenic effects of PFAS may be attenuated by exercise and diet [96]. There is less evidence for lipid subfractions *e.g.* apolipoprotein B (apoB) on chylomicrons, VLDL and LDL or apolipoprotein A1 (apoA1) on HDL [42]. Yet, these may provide a deeper understanding of underlying perturbations and may be better predictors for atherosclerosis (*i.e.* apoB is a direct measurement of the number of LDL particles and more, smaller LDL particles poses a greater risk than fewer, larger LDL particles) [97].

The evidence for associations with hard endpoints of obesity, T2D and CVD is more limited and inconsistent. For obesity in adolescents, an association between prenatal PFAS and lower weight during the first 2 years of life, but higher weight during childhood and adolescence has been suggested [98]. Postnatal PFAS and weight during childhood and adolescence show direct [99,100], null [100,101] and inverse [101-103] cross-sectional associations. For T2D, several prospective studies with direct [104,105], null [106,107] and borderline inverse [41] associations can be found in the literature. For CVD, due to i) the strong and well evidenced link between CVD and blood lipid levels and ii) the association between PFAS

and blood lipids, an association between PFAS and higher CVD risk can hypothetically be expected [58]. In fact, according to EFSA, a 5% increase in total cholesterol would result in, at least, a 5% increase in risk of CVD, which is clinically relevant [35]. Correspondingly, there are studies showing that PFAS is associated with markers of intima-media thickness (a measure of atherosclerosis) [108,109] and three cross-sectional studies [110–112] and one ecological study found direct associations [113] of PFAS with CVD. However, as mentioned above, other pathways may mediate the PFAS-CVD relationship in the other direction as well [114] and there are also findings of null or inverse associations, including prospective studies on coronary artery disease [87] and on stroke [115] in the C8 cohort, one nested case-control study [116] and one recent cross-sectional study on stroke [117]. In addition to inconsistencies in results, there are also methodological limitations (*i.e.* lack of temporality criterion, small sample sizes and self-reported endpoints and pooling of etiologically different CVD). Therefore, high-quality epidemiological studies investigating PFAS and CVD associations are lacking yet they are extremely important for risk assessments and regulatory decisions.

2.3.5 The 'exposome' concept and mixture studies

Humans are exposed to a mixture of exposures throughout their life (one study estimated there are over 350,000 chemicals on the market [118]). Yet, most research is based on single exposures, which neglects potential chemical interactions or confounding by highly correlated chemicals. The definition of the 'exposome', as proposed by Wild in 2005, is "encompassing all life-course environmental exposures (including lifestyle factors), from the prenatal period onwards" [119]. Thus, aiming to study mixtures of chemicals or, rather ambitiously, all exposures at the same time, would provide a more complex, but perhaps also more truthful picture. Yet as of now, studies on mixtures are relatively scarce with statistical tools still evolving [112,120–123] and 'exposome' studies are even more novel [124–127].

2.3.6 OMICs-based studies

In order to establish causality, it is equally important to understand the molecular pathways underlying PFAS associations [128]. The discrepancies between laboratory studies and human physiology make it difficult to investigate molecular pathways that link PFAS to adverse outcomes. Thus, we need insights from human studies using molecular markers. The use of OMICs data allows for comprehensive systemic approaches but brings along great challenges for complexity of analyses. OMICs data refers to big datasets including genomics, proteomics and metabolomics. These can be analyzed singularly or integrated into one pipeline (multi-OMICs). Genes are the hereditary units that are transcribed into RNA, which can be translated into protein. Proteins are large molecules with different functions such as enzymes catalyzing metabolic reactions. Metabolites are small molecules which can be endogenous (*e.g.* amino acids or lipids formed by metabolic reactions) or exogenous (*e.g.* diet, drugs or pollutants). Whilst genes,

RNA and proteins are still under regulatory processes, metabolites are the end-product, and they are therefore closely related to the phenotype. OMICs data can be used to connect exposures to molecular signatures and to adverse outcomes using the "meet-in-the-middle" concept, where molecular signatures are selected based on their associations with both exposures and outcomes [129]. Thereby we can explore potential underlying mechanisms as well as advance with exposome-based studies, as untargeted metabolomics can measure many exposures and biological response markers at the same time.

Thus far, some metabolomics studies have been performed with PFAS exposure in occupationally exposed [130], adults [131–133], children [134] and pregnant and early postpartum women [135]. A few also used latent variable analysis to relate PFAS exposure and metabolomics data to health outcomes in children such as glucose metabolism measurements among overweight children [136], severity of non-alcoholic fatty liver disease [137] and liver injury [138]. These studies mainly indicated perturbations with lipids, fatty acids and oxidative stress, some amino acids and dietary factors [139]. Additionally, PFAS exposure associated with anti-inflammatory proteins in a proteomics study [63] and with altered gene expression in cholesterol-related genes [51]. However, no studies have linked multiple POP exposures to multi-OMICs data and to cardiometabolic outcomes, which could increase the understanding of underlying molecular perturbations.

3 Research aims

The overall research question in this PhD thesis was whether the prevalent exposure to the widespread and highly persistent PFAS contributes to the development of metabolic perturbations and increases the risk of CVD in the general population. To address this, we established five specific aims:

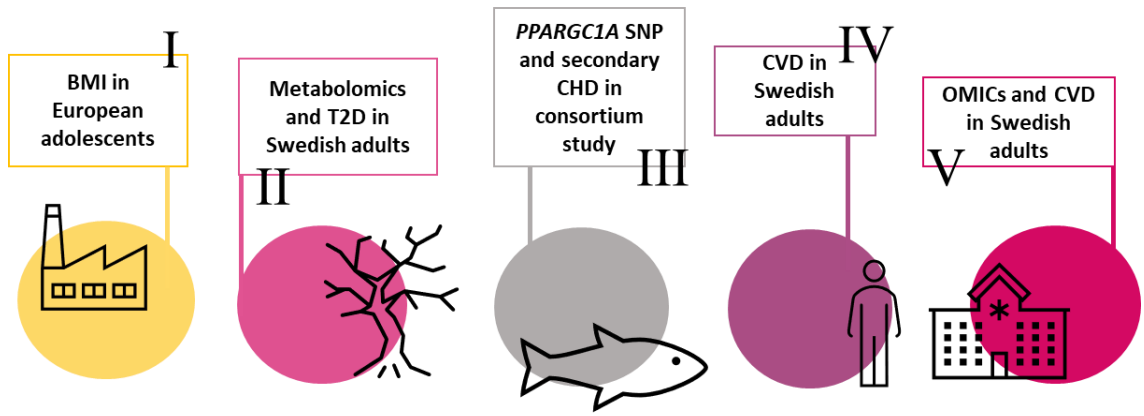
Paper I To assess associations between PFAS exposure and metabolic perturbations (BMI) in European adolescents.

Paper II To assess associations between PFAS exposure and metabolic perturbations (T2D) in adults – including untargeted metabolomics to improve mechanistic insight in PFAS affected pathways.

Paper III To investigate associations between genetic variants in the *PPARGC1A* gene, a coactivator of nuclear receptors potentially activated by PFAS exposure, and subsequent coronary heart disease.

Paper IV To assess associations between PFAS exposure and risk of incident myocardial infarction and stroke and its major intermediate risk markers.

Paper V To explore the underlying pathways linking multiple POP exposure (PFAS and OCs) to cardiometabolic perturbations – including genetics, proteomics and untargeted metabolomics data.



4 Materials and methods

4.1 Study participants and study design

4.1.1 Human Biomonitoring for Europe (HBM4EU)

The HBM4EU Aligned Studies are a survey aimed at collecting samples and data as harmonized as possible from European regional and national studies to derive current internal exposure data representative for the European population across a geographic spread [140,141]. We included studies in **Paper I** if PFAS measurements were available leading to a final study population of n=1,957. Studies were cross-sectional and were from Norway (NEBII), Sweden (Riksmaten Adolescents 2016-17), Slovakia (PCB cohort follow-up), Slovenia (SLO CRP), Greece (CROME), Spain (BEA), France (ESTEBAN), Belgium (FLESH IV) and Germany (GerES V-sub). For the outcome of BMI, length and weight were measured by nurse/physician or self-reported.

4.1.2 Västerbotten Intervention Program (VIP)

The VIP is a sub-cohort in the Northern Sweden Health and Disease Study initiated in 1985 [142]. Inhabitants within Västerbotten County (**Figure 5**) were invited to a health examination when they became 40, 50 or 60 years old, including a questionnaire on diet and lifestyle. The participation rate exceeded 56%, often around 70%. In **Paper II**, we used a nested case-control design and included VIP participants with diabetes (n=187) that had donated samples of blood to the biobank on at least two occasions (approximately 10 years apart), of which at least one occurred *prior* to T2D diagnosis. Cases of T2D were matched (1:1) according to gender, age, sample date (±90 days) at baseline examination with VIP participants without T2D (controls) that were alive at the time of T2D diagnosis for the corresponding case and had donated blood on two occasions. T2D cases were identified in the DiabNorth register [143], which was diagnosed by a physician and validated by autoantibodies. PFAS and lipids were measured from stored fasting plasma samples at baseline and follow-up.

Figure 5. Locations included in the Swedish studies (VIP, 60YO and SMC). (Source: Personal Collection)

4.1.3 Genetics of Subsequent Coronary Heart Disease (GENIUS-CHD)

This consortium aimed to investigate the impact of genetics on secondary CHD events and included studies of different epidemiological designs (mostly cohort studies) [144,145]. Studies included in our analyses for **Paper III** were from Austria, Canada, Finland, Italy, Germany, Netherlands, New Zealand, Scotland, United Kingdom and United States. Outcomes included secondary CHD events and all-cause mortality.

4.1.4 The Cohort of 60-Year-Olds (60YO)

The 60YO was established with the aim to study CVD etiology and randomly invited men and women aged 60 years from Stockholm County (**Figure 5**) for a baseline examination between 1997-99 (78% response rate, n=4,232). The study participants underwent a health examination, completed a questionnaire, and donated blood samples [146]. For **Paper IV**, we used a nested case-control design and selected first incident cases of primary myocardial infarction (n=214) and ischemic stroke (n=183). Each case was randomly matched (1:1) to a control, if alive and free from the case diagnosis at the time the case experienced the event, based on sex and sample date (±90 days). CVD cases were diagnosed by physicians and identified via linkage to the National Patient Register among patients free of prevalent CVD from baseline through 2014 (International Classification of Diseases (ICD), 10th Revision: I21 and I63 for myocardial infarction and stroke, respectively). PFAS and lipids were measured in fasting blood samples at baseline. Lipids were measured via automated hospital routines. For lipid analyses, only the controls that were not using lipid-lowering medication were used (n=305).

4.1.5 Swedish Mammography Cohort (SMC)

The SMC (part of SIMPLER, Swedish Infrastructure for Medical Population-based Life-course Environmental Research) was established between 1987 and 1990, when all 90,303 women who were born 1914-1948 and residing in two counties, *i.e.* Västmanland and Uppsala (**Figure 5**), in Central Sweden received a questionnaire concerning diet and anthropometry (74% response rate, n=61,433) [147]. A clinical sub-cohort (SMC-C) was established between 2003 and 2009 of women <85 years of age and living in Uppsala town and surrounding areas (61% response rate, n=5,022) and participants completed a questionnaire and donated blood samples. Additionally, data on genetics, cardiovascular proteins and untargeted metabolomics is available. For **Papers IV and V**, we used a nested case-control design and selected first incident cases of primary myocardial infarction (n=135) and ischemic stroke (n=173). Case-control pairs were randomly matched (1:2 for myocardial infarction and 1:1 for stroke), if alive and free from the case diagnosis at the time the case experienced the event, based on age (±1 year) and sample date (±90 days). CVD cases were diagnosed by physicians and identified via linkage to the National Patient Register among patients free of prevalent CVD from baseline through 2017 (International Classification of Diseases (ICD), 10th Revision: I21 and I63 for myocardial infarction and

stroke, respectively). PFAS and lipids were measured in fasting blood samples at baseline. Lipids were measured via automated hospital routines. For lipid analyses, only the controls that were not using lipid-lowering medication were used (n=326). For omics analyses in **Paper V**, only subjects with available omics data were used (n=657).

4.2 Ethical considerations

For the European study (HBM4EU) in **Paper I**, individual ethical approvals and informed consent were obtained in each cohort. Additionally, an ethical approval was obtained for data analysis in Sweden. For the consortium study (GENIUS-CHD) in **Paper III**, we have ethical approvals for the SHEEP study where we performed the analysis on individual data; the other studies participating in the consortium each had their own approval and we only obtained analysis results from each study. For the other population-based cohorts in Sweden (VIP, 60YO and SMC) used in **Papers II, IV and V**, we had ethical approval from the Regional Ethical Review Board at Karolinska Institutet (Stockholm, Sweden).

The handling of sensitive personal data was done in accordance with Good Data Protection Rules (GDPR). Thus, to protect the privacy of the participants, the ID numbers were pseudonymized and data is stored on secure servers (SecureLAN at Karolinska Institutet and SNIC-SENS at Uppmax, Uppsala) to which only the authors of the studies had access (the authors had no access to the pseudonymization key). As such, we take responsibility for the integrity and accuracy of the data analyses. Furthermore, to employ good and open research practice, the research was approved by Ethical committees *prior* to the start of the research, informed consent (oral/written) was obtained from all participants and the plan of analyses as well as analyses scripts and findings are documented using electronical laboratory notebooks.

4.3 Analytical methods and covariate assessment

4.3.1 PFAS measurement

PFAS were measured in blood samples at baseline (and at follow-up in Paper II) using targeted measurements. Quality control assessments were performed in each of the laboratories and most of them also participated in quality control assessment programs.

In **Paper I**, plasma or serum PFAS were measured using liquid chromatography tandem mass spectrometry or ultraperformance liquid chromatography-tandem mass spectrometry. This resulted in detectable levels for PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFHxS, PFHpS and PFOS in at least one of the studies. Limit of quantification (LOQ) ranged from 0.01 to 0.50 ng/mL and <LOQ was imputed using single random imputation from a truncated lognormal distribution in studies with at least 70% of values ≥LOQ. In **Paper II**, plasma PFAS was measured at the National Institute for Health and Welfare in Kuopio using targeted liquid chromatography-triple quadrupole mass

spectrometry [148]. There were detectable concentrations for PFHxS, PFOS, PFOA, PFNA, PFDA and PFUnDA. Data <LOQ (0.15 ng/mL) was replaced with LOQ divided by two in 26% and 42% for PFDA and PFUnDA, respectively. In **Papers IV and V**, serum PFAS was measured at the Division of Occupational and Environmental Medicine at Lund University using liquid chromatography-triple quadrupole linear ion trap mass spectrometry (QTRAP 5500, AB Sciex) using selected reaction monitoring in negative ion mode [149]. Limit of detection (LOD) ranged from 0.01 ng/mL for PFHpA to 0.09 ng/mL for PFOA and <LOD was replaced with LOD divided by the square root of two for <3% of samples for PFHpA and PFUnDA. Finally, PFHxS, PFOS, PFHpA, PFOA, PFNA, PFDA and PFUnDA were measurable, but PFOA and PFHpA were not further analysed in the SMC-C subjects as concentrations were remarkably high likely due to contamination.

4.3.2 OMICs measurement

In **Paper II**, untargeted liquid chromatography-quadrupole time of flight mass spectrometry (Agilent Technologies) metabolic profiling was performed on baseline and follow-up plasma samples [150,151]. Matched case-control pairs and repeated measurements were analysed in the same batch. Both reverse phase and hydrophilic interaction liquid chromatography columns in positive and negative ionization modes were used. Corrections and normalizations were performed (MassHunter Acquisition B.04.00 software, XCMS R package, batchCorr R package) [152,153]. Measurement drift per batch and quality control were monitored. Missing values were replaced with random values from a normal distribution between 0 and the lowest measured peak intensity for each feature and features with low retention time in hydrophilic interaction column were removed. Putatively annotated PFAS metabolite features were removed *prior* to final analytical modelling.

In **Paper V**, metabolomics, proteomics and genomics data were measured in the same fasting blood samples as used for the POP measurements in the SMC-C cohort. Untargeted metabolomics was performed using liquid chromatography-quadrupole time of flight mass spectrometry (Agilent Technologies). Reverse phase chromatography using C18 columns in positive and negative ionization modes were used [154]. Quality control samples were injected at the beginning, end and evenly throughout batch sequence. Corrections, normalizations, imputations and grouping of features derived from a single metabolite were performed (XCMS, IPO, MetNormalizer, StatTools and RAMClustR R packages). Features with a coefficient of variation >30% among quality control samples were removed. Putatively annotated PFAS features were removed *prior* to final modelling.

Proteins were measured using high-throughput multiplex immunoassays (Olink Proseek Multiplex CVDII, CVDIII and Metabolism) and provided normalized protein expression values on a log2 scale standardized per analysis plate. Proteins >25% <LOD were removed *prior* to analysis and missing values were imputed (StatTools R package).

Genotyping was performed using the Illumina GSAMD-24v1-0_20011747_A1 BeadChip, USA and SNPs were imputed up to Haplotype Reference Consortium (HRC) v1.1 and 1000 Genomes project phase 3. The results were then analysed using the software GenomeStudio 2.0.3 from Illumina, USA. The sample success rate was ≥98%. To prefilter the genetics data, we selected SNPs associated with POP exposures in a linear model with additive effects at an arbitrary cut-off of $p<0.000005$ using Plink 2.0 software.

4.3.3 Covariates (DAG approach)

We based our confounder selection on *a priori* assumptions of variables that theoretically impact both the exposure and the outcome and could thus confound associations [155]. The confounders included in the different papers are listed in **Table 1** and visualized in **Figure 6** [156]. The directed acyclic graph (DAG) indicates that the minimal sufficient adjustment set for testing associations between PFAS and outcomes (BMI, lipids, CVD and T2D) needs to contain age, sex, education and diet. Furthermore, analyses can be adjusted for physical activity and smoking status as they can strongly impact our outcomes as well as for sampling year, as ancestor of our exposure, and for dyslipidemia medication, diabetes, hypertension and family history of CVD, as ancestors of our outcome. Dyslipidemia medication may artificially impact lipid levels, thereby influencing associations between PFAS and lipids, whilst diabetes, hypertension and family history of CVD are strong risk factors for CVD. BMI and lipids could be mediators or confounders and we therefore investigate associations both with and without adjustment for them [157]. Additionally, we performed stratified analyses if there were suggested effect modifiers (*e.g.* sex and BMI).

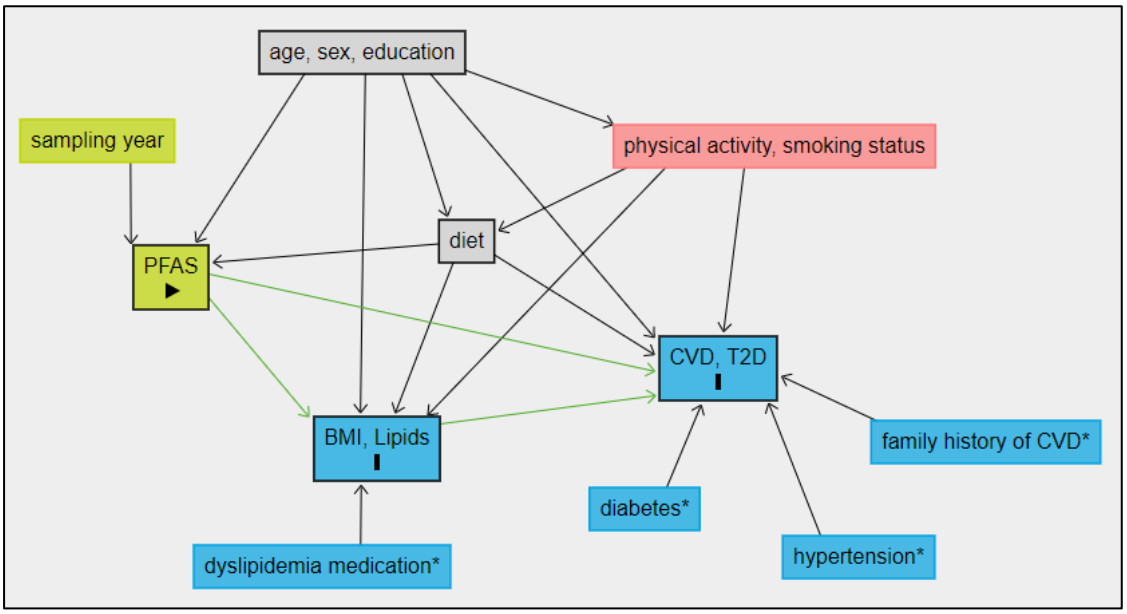


Figure 6. Directed acyclic graph. Boxes represent the exposure and ancestors of the exposure (green), confounders (red for unadjusted, grey for adjusted), outcomes, ancestors of the outcomes and mediators (blue). Arrows indicate open (representing statistical associations between variables; green) or closed (representing absence of associations; black) paths between variables. (Source: Created with DAGitty).

4.4 Statistical analysis

4.4.1 Classical approaches

To investigate statistical associations between exposures and outcomes, we used conditional logistic regression (for categorical outcomes in matched case-control studies), linear regression (for continuous outcomes), generalized equation modelling (for longitudinal data) and cox proportional hazards regression (for survival analysis), while adjusting for confounders. Exposures were analyzed per distribution-based tertiles or as continuous with log-transformation and standardization. Furthermore, **Papers I and IV**, included data from multiple studies and we used mixed effects models and random effects meta-analysis to be able to pool different studies whilst accounting for the variance between the different studies. Both approaches gave very similar results and we opted for mixed effects models if the heterogeneity between studies was small, whilst we show the random effects meta-analysis approach if the heterogeneity between studies was larger. Also, the meta-analysis approach provides a clear visual summary of individual study results. In **Paper III**, we used an inverse variance weighted fixed-effect meta-analysis model. This gives more weight to larger studies (with smaller standard error) and the fixed-effects model assumes one true estimate across all studies (in contrast to random effects, allowing it to vary between studies) [158].

4.4.2 Mixtures

In **Paper I** we performed mixture analysis using a quantile G-computation approach to find an overall mixture effect estimate as well as individual contributions of each compound to the mixture effect. This is a relatively novel method to estimate effects of an exposure mixture without assuming directional homogeneity of individual compounds [159]. First, exposures are transformed into quantized versions and a linear model is fitted which estimates the change in the outcome expected for a one-unit change in all exposures. Then, the weights of each exposure are calculated. Weights can be positive or negative in direction and the sum of weights in each direction equals to one. There are several tools for estimating mixture effects that each have their own strengths and limitations. Advantages of quantile G-computation are the simplicity of inference and implementation (compared to *e.g.* Bayesian Kernel Machine Regression) as well as allowing for directional heterogeneity (compared to *e.g.* Weighted Quantile Sum regression), whilst it is limited in exploring interactions between individual compounds at different exposure levels (high *vs* low) [160].

4.4.3 Machine learning

Whilst classical approaches are considered the gold standard in inference and modelling of pre-defined hypotheses, machine learning is often considered more predictive and data driven. Machine learning makes less assumptions about the data and underlying

hypotheses in advance. It works well with datasets with more variables than subjects, allows for implicit interactions in the data and avoids the need for large multiple testing adjustments. In our more exploratory OMICs analyses in **Papers II and V**, we therefore used machine learning to select OMICs variables related to exposures. On the downside, machine learning can be vulnerable to overfitting, more complex to interpret and does not allow for adjustment for confounders. Thus, we use an in-house developed random forest algorithm for predictive multivariate modelling with minimally biased variable selection incorporated into a repeated double cross-validation framework to minimize the overfitting ('MUVR') [161]. Subsequently, we followed up with classical approaches to adjust for confounders and filter out exposure-related OMICs variables that were likely selected due to confounding.

Finally, to facilitate interpretation of exposure-OMICs-outcome associations, we used an in-house developed visualization tool ('Triplot') [162]. This tool is based on principal component analysis (PCA), which is a data reduction technique reducing variables to a lower number of components while preserving as much of the data variance as possible. We also visualized networks of Spearman partial correlations between the selected OMICs features using a Gaussian Graphical Model of their respective Pairwise Markov Random Field (PMRF) models. This means that correlations between variables are adjusted for all other variables in the network. In this network, the nodes represent variables connected by undirected edges that can be interpreted as the partial correlation coefficients, shrunken by the Least Absolute Shrinkage and Selection Operator (LASSO) using the Extended Bayesian Information Criterion (EBIC) [163]. Communities were detected using the Spinglass algorithm [164,165]. The total pipeline for OMICs analyses is illustrated in **Figure 7**, whilst an overview of the different statistical methods used in the five studies included in this PhD project is presented in **Table 1**.

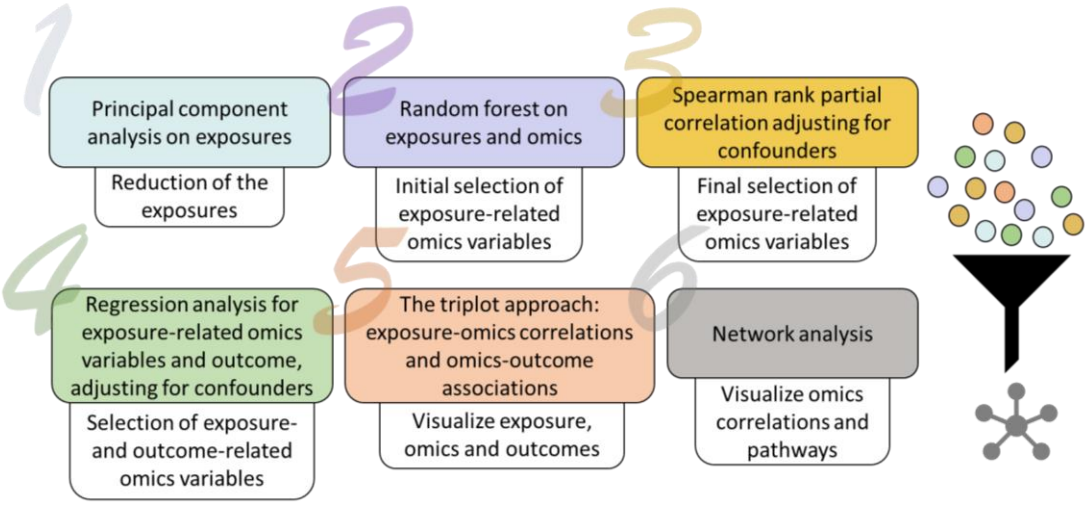


Figure 7. Overview of the approach used in OMICs studies included in this thesis. (Source: Personal Collection)

Table 1. Overview of designs and methods used in each study.

	Study I	Study II	Study III	Study IV	Study V
Population	HBM4EU European teenagers n=1,957	VIP Swedish adults n=374	GENIUS-CHD Global CVD survivors n=80,900	SMC-C & 60YO Swedish adults n=1,528	SMC-C Swedish adult women n=657
Design	Cross-sectional Meta-analysis	Prospective Nested case-control	Prospective Meta-analysis	Prospective (& cross-sectional) Nested case-control Meta-analysis	Prospective (& cross-sectional) Nested case-control
Outcome	BMI Measured or self-reported	T2D/blood lipids Register-linkage/measured	Subsequent CVD Diagnosed	CVD/MI/stroke /blood lipids Register-linkage Measured	CVD/MI/stroke /blood lipids Register-linkage Measured
Exposure	PFAS Measured	PFAS & OMICs Measured	*PPARGC1A* SNPs Measured	PFAS Measured	POP & OMICs Measured
Covariates of adjustment	Age, sex, study, (education household, fish intake)	Age, sex, sample year, marital status, education, smoking status, physical activity, (fish, meat and alcohol intake and BMI)	Age and sex	Age, sex, sample year, (education, BMI, diabetes, hypertension, family history of CVD, smoking habits, physical activity and healthy diet score)	Age, sample year education, family history of CVD, smoking habits, physical activity, healthy diet score (BMI, LDL, HDL, triglycerides and hypertension)
Statistical analysis	Linear regression Mixed effects models/ Meta-analysis/ Mixture G-comp.	Conditional linear/logistic regression Random forest, partial Spearman correlation, triplot	Cox proportional hazards regression Meta-analysis Additive genetic model	Conditional logistic/linear regression Mixed effects models/Meta-analysis	Conditional logistic/linear regression Additive genetic model Random forest, partial Spearman correlation, triplot, network

5 Results

5.1 PFAS and BMI in European teenagers (Paper I)

The cross-sectional analysis included 1,957 teenagers (ages 12-18 years) from nine European countries. The most prevalent PFAS in blood were PFOA, PFNA, PFHxS and PFOS and we observed generally higher PFAS levels in North and West regions of Europe compared to East and South. Medians for PFOA ranged from 0.66 to 1.47 ng/mL within studies. Our main analysis indicated an overall inverse association between PFAS and BMI, which was stronger in boys. This was significant for PFOA where an increase from the 25th to the 75th percentile in PFOA associated with a decrease of 0.08 in age-and sex-adjusted BMI z-score. Stronger inverse associations were observed in boys than in girls and similar tendencies for inverse associations were found for PFAS with overweight/obese. Adjustment for socio-economic status, fish intake, breastfeeding and birthweight only marginally impacted models. Additional adjustment for dietary variables (eggs, milk, meat, fastfood), degree of urbanization, sampling season or removal of self-reported BMI subjects did not impact model estimates and were not included in final models. There was some heterogeneity between studies, which could be related to differences in ages (puberty) or PFAS levels between studies.

Mixture analysis indicated that the mixture [PFOA, PFNA, PFHxS and PFOS] also associated inversely with BMI z-score with an estimate of -0.05 (-0.13, 0.03) per one quartile increase in PFAS mixture and that PFHxS contributed opposite to PFOA, PFNA and PFOS (**Figure 8**). Associations of PFHxS with BMI z-score adjusted for [PFOA, PFNA and PFOS] indicated stronger positive associations (estimate moved from 0.01 to 0.06 in linear mixed effects models). This indicates that different PFAS compounds may have diverging effects on BMI. In conclusion, the observed associations between PFAS and lower BMI in this study seem to be driven by PFOS and PFOA, whilst PFHxS may associate with higher BMI.

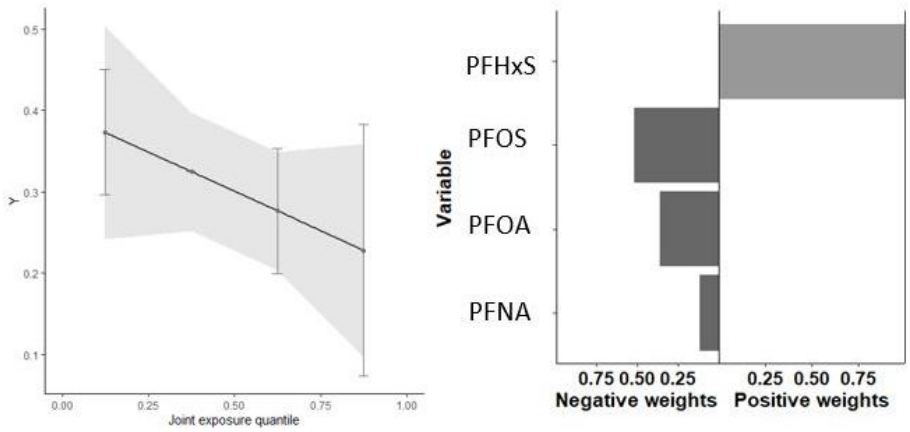


Figure 8. Visualization of quantile G-computation results showing associations of PFAS mixture [PFHxS, PFOS, PFOA and PFNA] with BMI z-score in teenagers (left) and contributions of each individual PFAS to the mixture effect (right). The length of the bars corresponds to the effect size relative to the others in the same direction, whilst the darkness of the bar corresponds to the overall effect size. (Source: Schillemans et al. 2022, *Environmental Pollution*)

5.2 PFAS, metabolomics and T2D risk in Swedish adults (Paper II)

A previous study, using the same nested case-control study on T2D in the VIP cohort, found a tendency for inverse associations of PFAS with T2D risk (OR 0.52, 95% CI: 0.20, 1.36 comparing the third with the first tertile for the sum of PFAS [PFOS, PFOA, PFHxS, PFNA, PFDA and PFUnDA]) [41] as well as with triglycerides, but not with cholesterol or hypertension [91]. In this follow-up study, we then included metabolomics data and aimed to shed light on molecular pathways underlying the observed inverse associations.

We found 290 PFAS-related metabolite features in the random forest model, of which 171 were significantly correlated with PFAS levels even after adjustment for confounders and multiple testing. Out of these, 35 also associated with T2D risk after adjustment for confounders. For an overview, we performed a PCA on the metabolite features and visualized the first two components in a Triplot. This shows the loadings of metabolite features in each component as well as the associations of components with exposures and outcomes (**Figure 9**). We found several glycerophospholipids that correlated positively with longer chain PFAS and associated inversely with risk for T2D. In addition, we found several diacylglycerols, which also correlated positively with longer chain PFAS, but they associated with increased risk for T2D. These results indicate that PFAS, particularly the longer chain (PFNA, PFDA and PFUnDA), associate with two groups of lipid species with opposite relations to T2D risk.

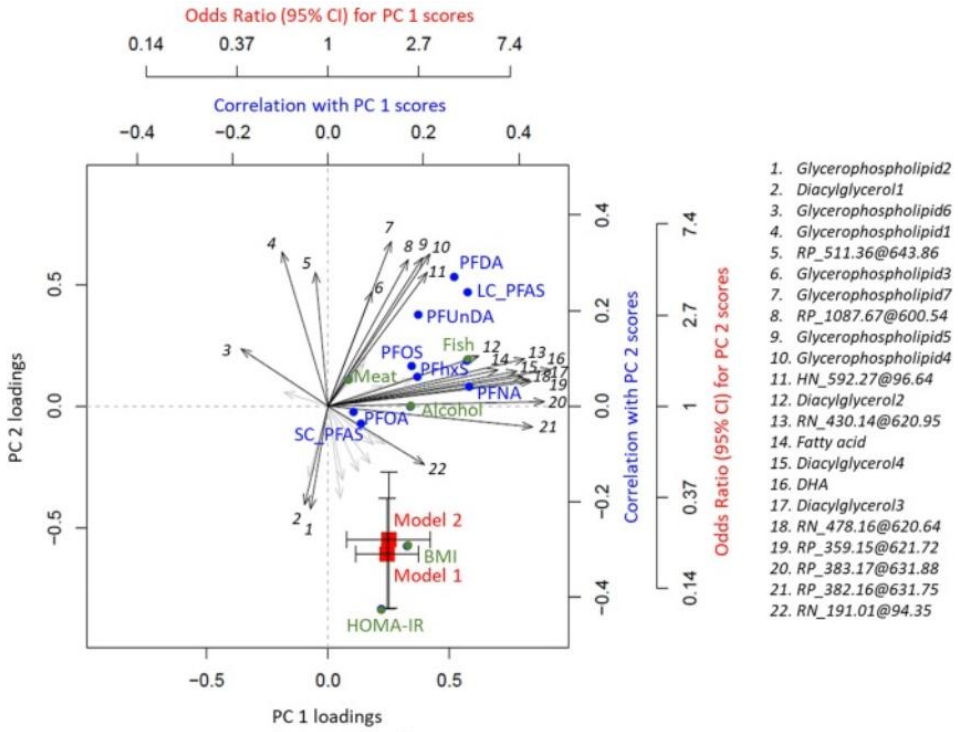


Figure 9. Triplot visualizing PFAS- and T2D-related metabolite features and their associations with longer (LC) and shorter chain (SC) PFAS, fish, meat, alcohol, BMI, HOMA-IR and T2D risk. Features, exposures and outcomes pulling in the same direction are associated, whilst opposite directions indicate inverse associations. Thus, horizontal features (right) associate with LC_PFAS (right) and T2D risk (right), whilst vertical features (top) also associate with LC_PFAS (top), but inversely with T2D risk (bottom). (Source: Schillemans et al. 2021, *Environment International*)

In another study not included in this thesis [166], we investigated those 290 PFAS-related metabolite features for their associations with blood lipids using the control subjects of the T2D study. Similarly, glycerophospholipids correlated with longer chain PFAS and associated inversely with triglycerides, after adjustment for confounders. However, we found no significant associations between the PFAS-related features and cholesterol. Interesting to note is that the PFAS-and triglyceride-related metabolite pattern followed the longer chain PFAS pattern (PFNA, PFDA and PFUnDA) over time (**Figure 11**).

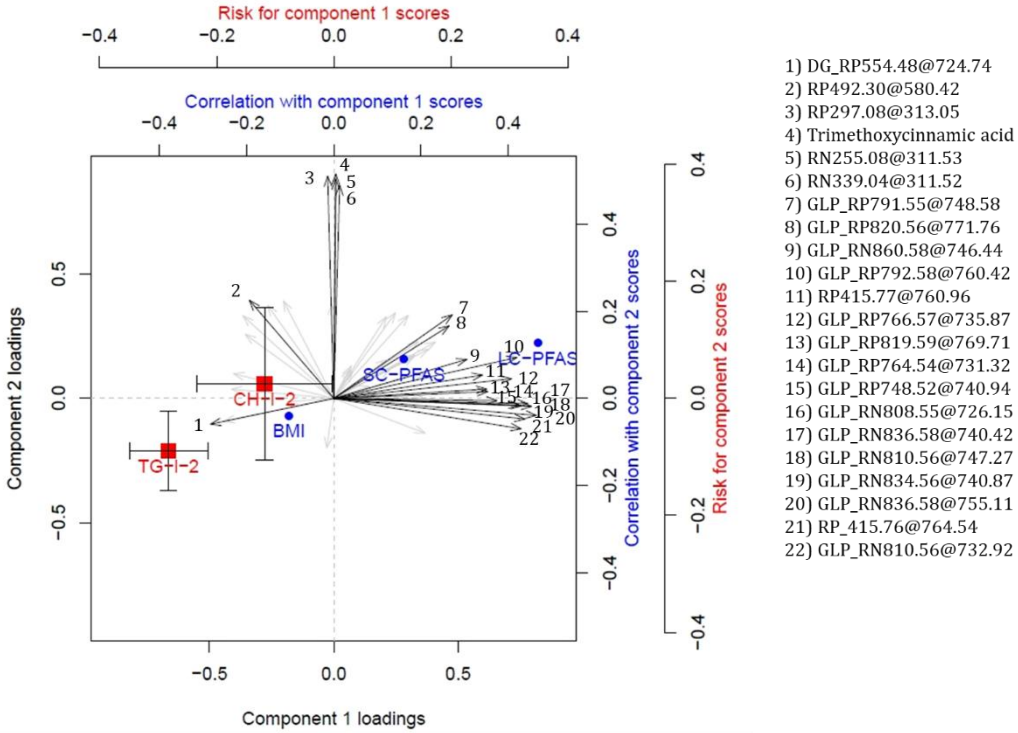


Figure 10. Triplot visualizing PFAS- and triglyceride-related metabolite features and their correlations with longer chain (LC) and shorter chain (SC) PFAS and BMI as well as their associations with triglycerides and cholesterol. (Source: Schillemans et al. 2022, *Environmental Research*)

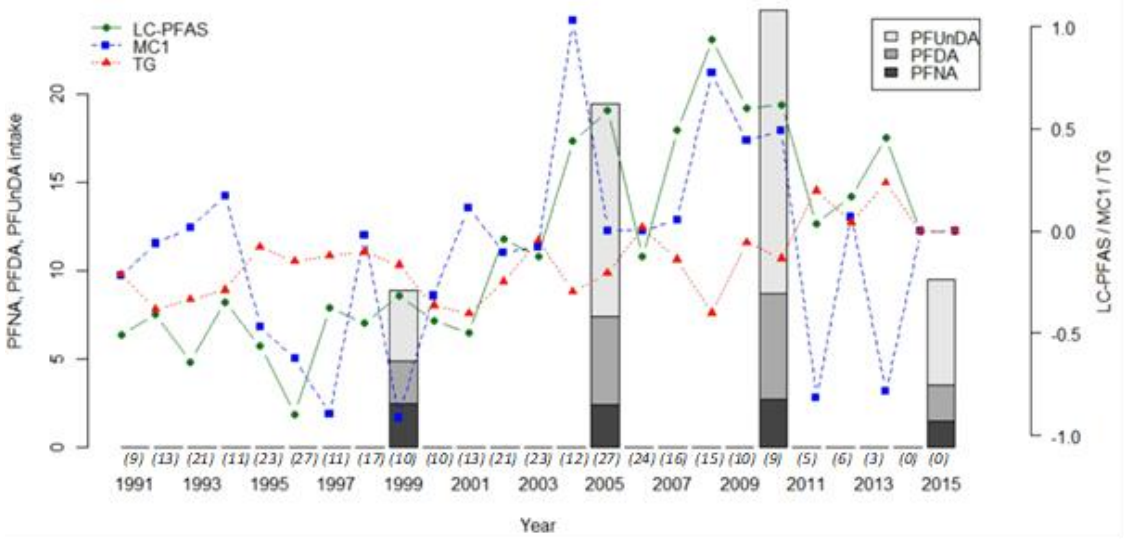


Figure 11. Temporal trends visualizing intake of PFAS (bars; median per calendar year in ng/day, adapted from Swedish Market Basket Survey 2015, National Food Agency rapport nr 26/2017) and sex- and birthyear-standardized medians of longer chain PFAS scores (LC-PFAS, green points and line), metabolite component scores (MC1, blue squares and dash) and triglyceride levels (TG, red triangles and dots) per year. (Source: Schillemans et al. 2022, *Environmental Research*)

5.3 Polymorphisms in *PPARGC1A* and secondary CVD events (Paper III)

We investigated polymorphisms in the *PPARGC1A* gene to gain insight in potential pathways relevant for CVD, which might also be relevant targets for PFAS exposure. Three SNPs were included based on literature-suggested involvement in cardiometabolic phenotypes, particularly rs8192678 (G482S). Results from the meta-analysis including 23 cohort studies within the GENIUS-CHD consortium indicated overall null associations between SNPs in the *PPARGC1A* gene and secondary events (**Figure 12**).

However, there was a tendency for an inverse association between rs7672915 (intron 2) with the main outcome of secondary CHD death or myocardial infarction (Figure 2 in paper: HR=0.97, 95%CI=0.94,1.00), which was significant for certain stratified analyses in vulnerable subgroups (older age, kidney disease, anti-platelet users, less than five years of follow-up). Other stratified analyses (sex, hypertension, T2D, BMI, statin use and left-ventricular impairment) were not significant. Sensitivity analyses (exclusion of cohorts deviating from Hardy-Weinberg Equilibrium, stratification by European ancestry) only marginally changed results.

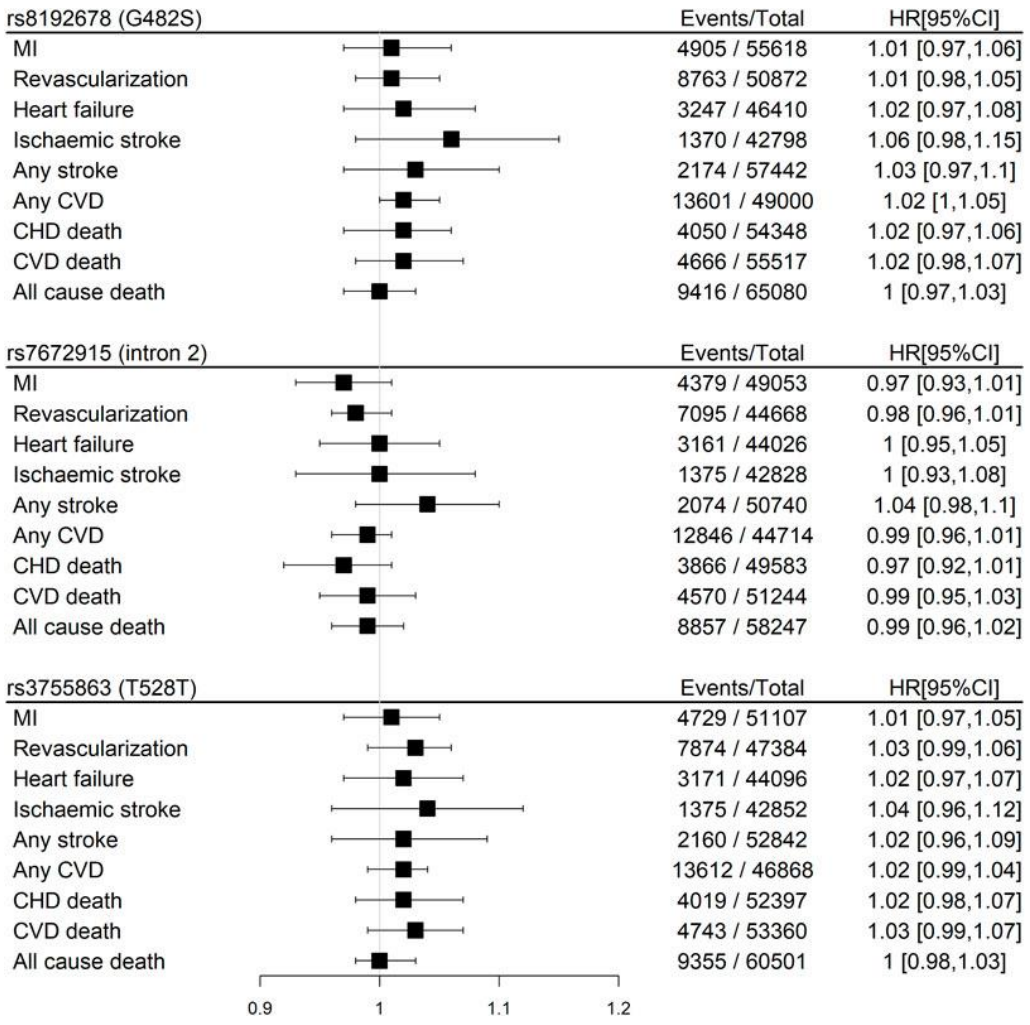


Figure 12. Forest plot visualizes overview of hazard ratios (HR) from meta-analysis for three SNPs in *PPARGC1A* with different CVD outcomes adjusted for age and sex. (Source: Schillemans et al. 2022, *Frontiers of Physiology*).

5.4 PFAS, blood lipids and CVD risk in Swedish adults (Paper IV)

Our main analysis included n=1,528 subjects pooling two Swedish cohorts (SMC-C and 60YO). PFAS levels were slightly higher in the SMC-C compared to the 60YO, except for PFOS, which was higher in the 60YO compared to SMC-C.

The findings showed inverse associations between PFAS exposure and increased CVD risk (composite of myocardial infarction and stroke: OR=0.70, 95%CI=0.53-0.93 for the standardized sum Σ PFAS [PFHxS, PFOS, PFNA, PFDA and PFUnDA]) when pooling the SMC-C and 60YO cohorts. Similar associations were found in individual cohorts. However, slightly more heterogeneity was observed between cohorts when separating analyses for stroke and myocardial infarction. Thus, results were obtained using a random-effects meta-analysis to pool cohorts. Associations for myocardial infarction risk remained inverse (OR=0.60, 95%CI=0.39-0.92 for Σ PFAS), whilst for stroke risk we observed a null association (OR=0.83, 95%CI=0.46-1.50 for Σ PFAS). It is worth noting that PFOS (and PFHxS in MI) showed the highest heterogeneity between studies. PFOS associated significantly with stroke, but inversely with MI in the SMC-C cohort and this same pattern was seen mirrored in the 60YO cohort. Associations were not materially different upon inclusion of fish intake as covariate.

As subsidiary analyses, we also assessed PFAS associations with blood lipids in controls who were not using cholesterol-lowering medication. PFAS associated with higher levels of total-, LDL-cholesterol but not with apoB. They also associated with higher levels of HDL-cholesterol, apoA1 and with lower levels of triglycerides. Associations with LDL and apoB were stronger among overweight/obese. The inclusion of blood lipids as covariates in the PFAS and CVD analyses did not impact the estimates, indicating that blood lipids were not responsible for the PFAS-CVD associations. Estimates are presented in **Table 2**.

Table 2. Multivariable-adjusted associations between baseline plasma PFAS and baseline blood lipids and CVD risk during follow-up in two Swedish pooled cohorts (SMC-C and 60YO cohort).

Σ PFAS T3 *vs* T1	**β-coefficient (95% Confidence Interval)**	**Σ PFAS T3 *vs* T1**	**Odds Ratio (95% Confidence Interval)**
Total Cholesterol	0.34 (0.15,0.53)	Myocardial infarction	0.60 (0.39,0.92)
LDL	0.26 (0.09,0.43)	Stroke	0.83 (0.46,1.50)
HDL	0.14 (0.07,0.20)	Composite	0.70 (0.53,0.93)
Triglycerides	-0.24 (-0.38,-0.10)		

Note: PFAS (PFHxS, PFOS, PFNA, PFDA and PFUnDA) were standardized (rescaled with mean=0 and SD=1) and summed (ΣPFAS). Results are presented for Tertile 3 (using Tertile 1 as reference) and models were adjusted for age, sex, sampling date, education, BMI, diabetes, hypertension, family history of CVD, smoking habits, physical activity, and healthy diet score. (Source: Adapted from Schillemans et al. 2022, *Environmental health Perspectives*).

As additional information in this thesis, we performed a mixture analysis using quantile g-computation for five PFAS and CVD (myocardial infarction and stroke) in SMC-C and 60YO (not included in the paper). This indicated also an inverse association between the PFAS mixture and CVD (OR=0.81, 95%CI=0.68-0.97) and individual PFAS contributions to the results indicated that these inverse associations were mainly driven by PFUnDA and PFDA whilst PFOS and PFNA contributed in the opposite direction and with minimal contribution from PFHxS (**Figure 13**). These results were in line with the regression results for individual PFAS.

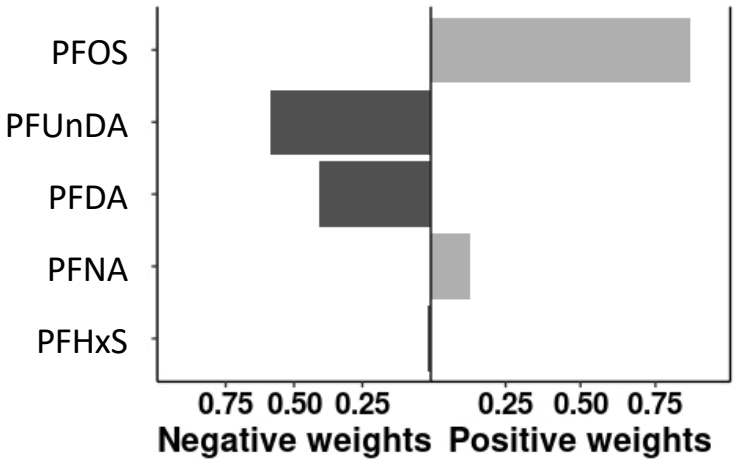


Figure 13. Contributions of individual PFAS to the PFAS mixture resulting from the quantile G-computation. The PFAS mixture associated inversely with CVD risk (OR=0.81, 95%CI=0.68-0.97). The length of the bars only corresponds to the effect size relative to the others in the same direction, whilst the darkness of the bar corresponds to the overall effect size. (Source: Personal collection).

5.5 POPs, OMICs and CVD risk in Swedish adults (Paper V)

The final study was based on the same nested case-control study on myocardial infarction and stroke in the SMC-C and aimed to investigate molecular pathways underlying the PFAS-CVD associations. We furthermore included OC exposures to be able to look at a broader group of POPs. We found 12 metabolite features that associated with both PFAS exposure and with one of the CVD outcomes (myocardial infarction, stroke or their composite endpoint). We also found 29 features (7 proteins and 22 metabolites) that associated with both OC exposure and with one of the CVD outcomes. All features were unique for either PFAS or OC and we found no genetic polymorphisms to be associated with both POP exposures and CVD.

PFAS-related features were glycerophospholipids, cortolone-3-glucuronide, exogenous synthetic chemicals and unknown metabolite features, whilst OC-related variables were proteins related to metabolism, mitochondrial and inflammatory pathways, carnitines, glycerophospholipids, mono-, di- and triglycerides, and hydroxy-DHA **(Figure 14)**. A PCA on the 41 POP- and CVD-related OMICs features showed 1) one component associated with increased risk of myocardial infarction, which correlated positively with OC and BMI and negatively with PFAS and 2) the second component associated with increased risk of stroke, which correlated positively with age and OC although the latter was diluted after adjustment for age (**Figure 15**).

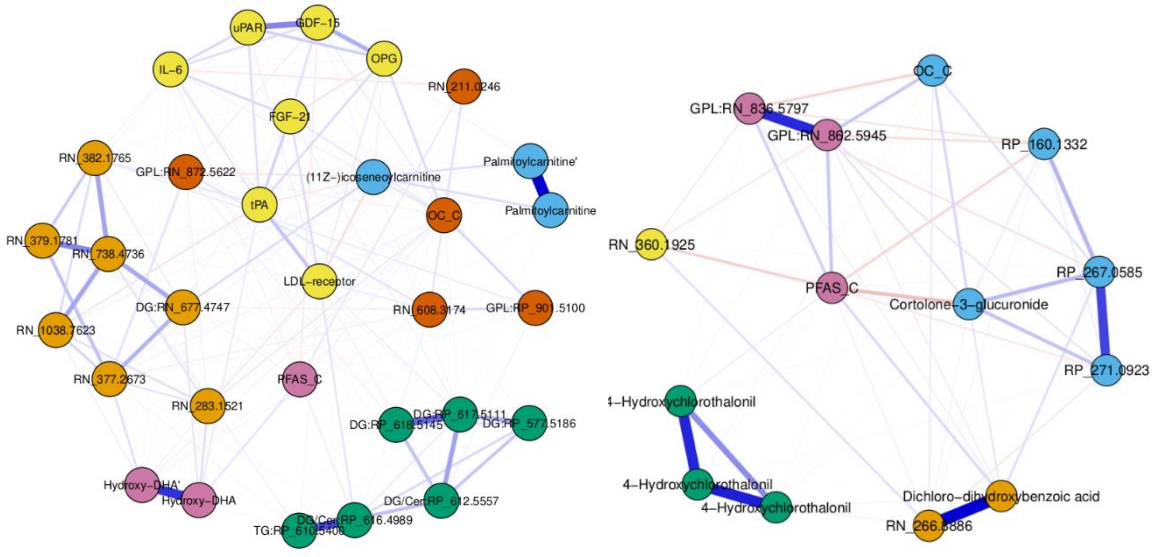


Figure 14. Estimated network structure of Gaussian Graphical Model with partial Spearman correlation coefficients of OC- and CVD-related OMICs features (left) and PFAS- and CVD-related OMICs features (right). Blue (positive) and red (negative) lines indicate partial correlations between features (adjusted for all other features in the model). Node colors represent clusters of features (obtained with Spinglass algorithm). (Source: Personal collection)

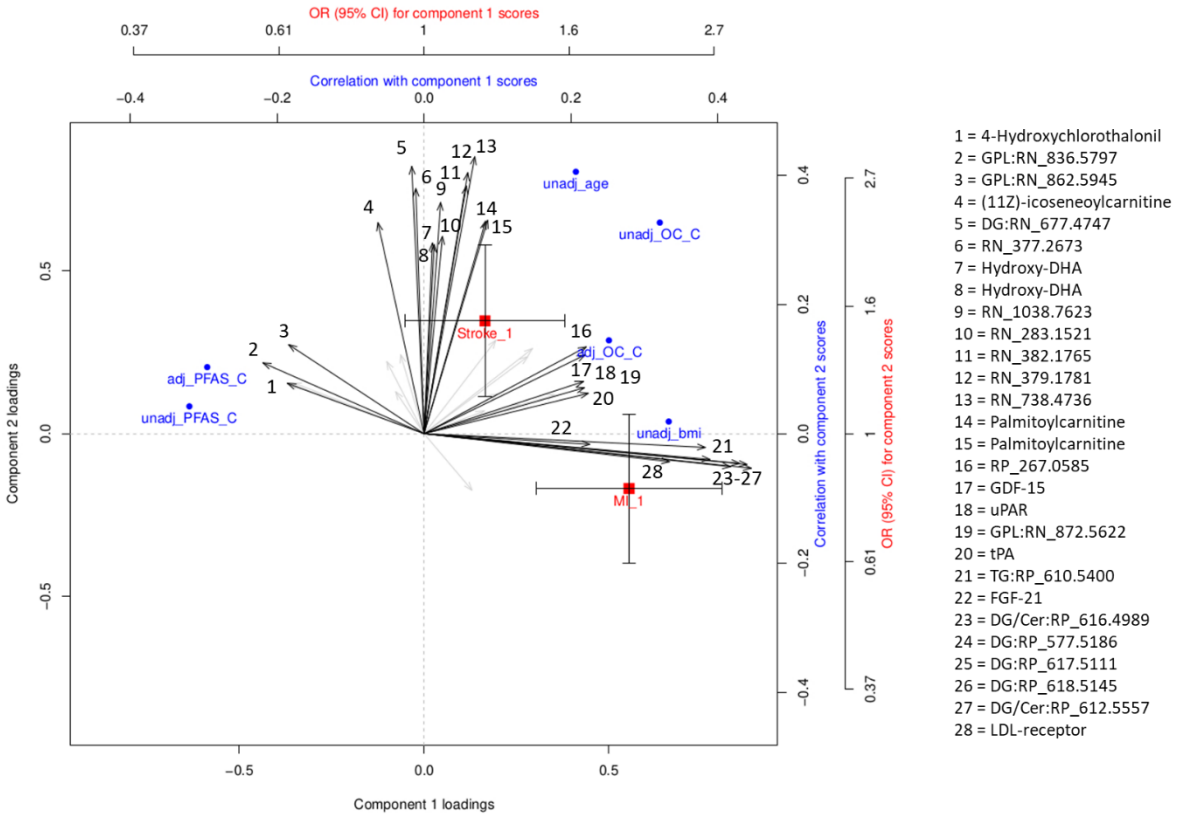


Figure 15. Triplot visualizing principal components of POP- and CVD-related OMICs features and their adjusted and unadjusted correlations with PFAS and OCs and unadjusted correlations with age and BMI as well as their adjusted associations with risk of myocardial infarction (MI) and stroke. Features, exposures and outcomes pulling in the same direction are associated, whilst opposite directions indicate inverse associations. Thus, features on the horizontal axis (right) associate with OC and BMI (right) and with MI risk (right), but negatively with PFAS (left). Features on the vertical axis (top) associate with OC (only weakly after adjustment), PFAS (only weakly) and age (top) and with stroke risk (top). (Source: Personal collection)

6 Discussion

6.1 Summary of the findings

This PhD thesis presents five epidemiological studies investigating persistent environmental contaminants in relation to cardiometabolic health to understand whether these pollutants increase the risk of CVD in the general population. The resulting findings indicate that low-level PFAS exposure is not associated with increased risk of cardiometabolic disease (BMI in adolescents, T2D, myocardial infarction and stroke in adults). However, results do indicate disturbances in lipid and inflammatory pathways, which may still affect human physiology as well as disease pathogenesis.

Paper I) PFAS associated with lower BMI in teenagers. Results implied this was driven by PFOS and PFOA, whilst PFHxS contributed in the opposite way (higher BMI).

Paper II) PFAS-related metabolite features aggregated in two patterns with opposite associations with T2D risk in adults. Longer-chain PFAS had stronger associations with metabolite features than shorter-chain PFAS. Associations between PFAS and T2D may be mediated by diacylglycerols and glycerophospholipids with opposite effects on T2D, *i.e.* diacylglycerol- and glycerophospholipid-related patterns associated with higher and lower T2D risk, respectively.

Paper III) SNPs in the *PPARGC1A* gene are not associated with secondary CHD. PPARGC1A is a master regulator of many metabolic pathways and several of these have been suggested as targets for environmental pollutants. Our findings however did not provide evidence for important roles of these pathways in CHD progression.

Paper IV) PFAS associated with lower risk of myocardial infarction but not with stroke. In line, we found associations with lower triglycerides and higher HDL-cholesterol, but on the other hand, we also found associations with higher total- and LDL-cholesterol. These results suggest that PFAS associations with HDL-cholesterol and triglycerides as well as a potential effect of PFAS on inflammation mediating the PFAS-CVD relationship should be considered more closely.

Paper V) PFAS-related OMICs features associated with lower risk of myocardial infarction, but not with risk of stroke whilst OC-related OMICs features associated with higher risk of myocardial infarction and stroke. This indicates that POP-CVD associations may be mediated via lipid (especially related to triglycerides), mitochondrial and inflammatory pathways. These OMICs patterns also correlated with age and BMI and metabolic markers for PFAS & OC exposures included other synthetic pollutants and potential markers for food intakes (*i.e.* fish, red meat and dairy) and microbiome diversity.

6.2 Scientific context and biological plausibility

6.2.1 Epidemiological evidence

The current body of evidence for associations between PFAS and cardiometabolic diseases in humans is rather inconsistent, but elevated cholesterol has been proposed as one of the main adverse events and this is a risk factor for CVD [35,45]. In one of our studies, we found associations of PFAS with elevated cholesterol in conjunction with lower myocardial infarction risk. This may indicate that pathways other than elevated cholesterol, like reduced triglycerides, may be more relevant for PFAS and CVD risk [167]. Our findings are in line with several other epidemiological studies indicating inverse associations with BMI in adolescents [101–103], triglycerides [168], T2D [106,169,170] and CVD [87,116,117]. However, there are also other findings of positive associations between PFAS and BMI in adolescents [99,100], triglycerides, T2D [104,105,171] and CVD [110,111,113]. A meta-analysis on published studies for PFOA and CVD indicates overall null associations with a more inverse tendency (**Figure 16**).

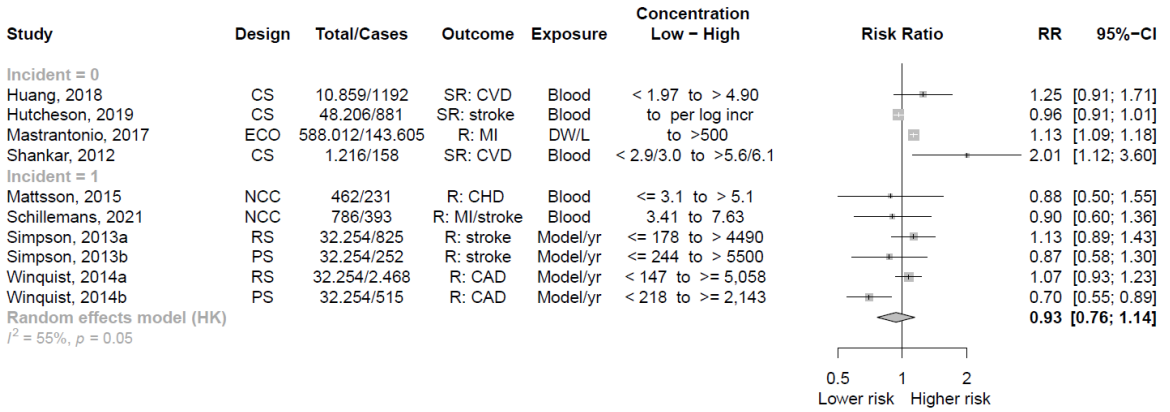


Figure 16. Forest plot showing results of a meta-analysis on associations of PFOA (in ng/mL) with CVD outcomes. Findings are separated by prevalent and incident CVD and overall result is shown for incident findings alone. Schillemans, 2021 only contains results from 60YO as PFOA was not available in SMC-C. Abbreviations: CAD, coronary artery disease; CHD, coronary heart disease; CVD, cardiovascular disease; DW/L, drinking water per liter; ECO, ecological; CS, cross-sectional; MI, myocardial infarction; NCC, nested case control; PS, prospective; R, register-based; RS, retrospective; SR, self-reported. (Source: Personal collection).

There are several potential explanations for these diverging results: First, it is possible that different PFAS concentrations may have different effects if there is a nonmonotonic dose-response relationship. The PFAS levels observed in **Paper I–V** correspond to low-level exposure (approximating lower benchmark doses [172], but still comparable to other general populations worldwide [45]) and associations may not be the same in populations highly exposed to PFAS. However, several studies from high contamination areas have similar findings of inverse associations [87,103,117]. Second, mixture effects could be distinct from individual effects [173] as multiple exposures could affect different [131] or the same [126] biochemical pathways in congruent or contrasting manner. This is also suggested by our results in **Paper I** where PFHxS counter-contributed to the PFAS mixture and in **Paper V** where PFAS correlated negatively also with OC-related OMICs features. Exposome-

related studies might shed light on effects within a broader spectrum of exposures and these have indicated similar findings of inverse associations of PFAS in relation to BMI and accrual of lean mass [123,124]. Third, we presented in **Paper II** that there may be different molecular features with similar exposure correlations but with opposite disease risk associations (diacylglycerols-higher T2D risk *vs* glycerophospholipids-lower T2D risk). This may contribute to conflicting overall findings. Fourth, effect modification by third variables, such as sex [103], BMI and dietary habits [82,93] or prenatal exposures [173], has been suggested to induce variability in results. Similarly, we found different associations in stratified analyses by sex in **Paper I** and by BMI in **Paper IV**. Lastly, there are other alternative explanations related to study limitations, such as reverse causation and confounding, which are further discussed in the methodological considerations section.

Our findings of OC-related OMICs features associating with increased risk of myocardial infarction and stroke are in line with most of the literature indicating associations of OC compounds with increased cardiovascular risk factors [174] and cardiovascular disease risk [175–180]. However, our studies show two important considerations: first, the strong impact of age on OC exposure and OMICs features even after adjustment for age in the models and second, the dilemma of BMI/lipids as a mediator or confounder (discussed further in the methodological considerations section).

6.2.2 Potential molecular mechanisms

Although most evidence for molecular mechanisms comes from animal or *in vitro* studies, there are also several human studies using OMICs data to investigate potential mechanistic pathways related to PFAS exposure and cardiometabolic diseases. Findings from OMICs studies included in this thesis (**Paper II and V**) indicated associations of POPs with several metabolite classes, *i.e.* diacylglycerols, trigycerides, glycerophospholipids, carnitines and other exogenous metabolites (pesticide and food metabolites) as well as with several proteins, *i.e.* LDL-receptor, fibroblast growth factor 21 (FGF-21), growth differentiation factor 15 (GDF-15), urokinase receptor (uPAR), tissue plasminogen activator (tPA) and interleukin 6 (IL-6). These results indicate potential perturbations in lipid (via diacylglycerols, trigycerides, glycerophospholipids, LDL-receptor, FGF-21), mitochondrial (via carnitines, GDF-15) and inflammatory (via GDF-15, uPAR, tPA, IL-6) pathways. This is in line with several other studies using OMICs [126], which indicated lipid metabolism [136,137], mitochondrial disruptions like fatty acid oxidation [131,132] and the carnitine shuttle [135] as well as anti-inflammation pathways [63] for PFAS and also lipids and fatty acids [181–183] for OCs.

For PFAS, these molecular markers match with activation of PPARα, which could lead to lower triglyceride levels and lower T2D as well as myocardial infarction risk. Contrarily, we did not find evidence for involvement of *PPARGC1A* polymorphisms in secondary CHD and this is a master regulator of metabolism and inflammation via regulation of NRs such as PPARα [72]. Nevertheless, this does not exclude a role for PPARGC1A-related pathways in

first event CVD development. Besides from glycerophospholipids and several carnitine-related metabolites (*i.e.* gamma-butyrobetaine, carnitine 13:0 and carnitine 13:1) linking PFAS to lower triglyceride levels and lower T2D risk, we also found diacylglycerols linking PFAS to higher T2D risk. This may be due to adverse effects related to PPARα activation or due to involvement of other NRs or pathways. We found no associations between PFAS and stroke risk and the correlations of PFAS with other carnitines (*i.e.* palmitoylcarnitine and 11Z-icosenoylcarnitine) associated with increased risk of stroke were only weak, but PFAS was linked to lower myocardial infarction risk via glycerophospholipids, FGF-21, GDF-15, tPA, LDL-receptor and triglycerides. These proteins are interconnected via metabolism, inflammation and endothelial function [184–187]. Moreover, triglycerides play an important role in atherosclerosis and this is a plausible connection between PFAS exposure and myocardial infarction [188]. However, when we adjusted our PFAS-CVD analysis for triglyceride levels (**Paper IV**), the estimates were not strongly impacted, which suggested potential involvement of other pathways. Additionally, the proteins and triglycerides were initially selected for their associations with OC and not PFAS exposure.

Molecular markers linking OC exposures to CVD correlated highly with age and BMI. Both of these have been suggested before as important factors that should not simply be looked at as solely confounders, but also as innate to the causal structure of the OC-CVD relationship [189]. Therefore, simple adjustment could remove too much of the OC overall effect. The OC-myocardial infarction relationship may be mediated by triglycerides, whilst OC-stroke may be mediated by carnitines. This could potentially be linked to activation of the aryl hydrocarbon receptor, as this receptor has been implicated in relation to OCs [190,191] before.

We found several exogenous compounds in **Paper V** that linked POPs to CVD risk, which could be related to shared sources (*i.e.* fish, red meat and dairy intake) [192] or be related to microbiome diversity [193,194]. The gut microbiome has been found to influence PCB metabolite levels in mice [195] and has also been suggested to mediate PFAS-metabolic effects [70]. On the one hand, we found evidence for associations between PFAS levels and lower triglyceride levels (potentially via PPARα activation). On the other hand, we did not find evidence for associations of PFAS with bile acids or molecular features underlying the association between PFAS and cholesterol, potentially because the inverse associations with triglycerides were stronger. We also found no evidence of associations for genetic polymorphisms linking POP exposures to CVD outcomes, despite literature findings having shown interactions between PFAS and genetic polymorphisms [51]. It is possible that genetic polymorphisms were not selected in **Paper V** as metabolite features and proteins are closer to downstream phenotypes and were thus more relevant for associations with POP exposures or due to methodological limitations (*e.g.* limited sample size and selection of SNPs related to POP blood levels, which could miss SNPs that are not related to POP blood levels but are relevant for POP-CVD associations).

6.3 Methodological considerations

6.3.1 Causal inference and study design

The objective of this thesis was to investigate whether POPs, especially PFAS, increase the risk of cardiometabolic diseases. For causal inference there are three imperative conditions: covariance, temporal precedence and ruling out of plausible rival explanations. Experimental studies (*e.g.* randomized control trials) generally provide stronger causal evidence as there is tight control over study protocols and variables [196]. However, experimental studies are not always applicable due to practical or ethical reasons and then observational studies are used. In this case, estimates reflect associations rather than causations and they have several potential limitations that need to be considered. The main issues are reverse causation, systematic error induced by bias or confounding, random error induced by small sample size or multiple testing and the generalizability of the findings [197].

6.3.2 Reverse causation

Reverse causation refers to the possibility that the outcome may have influenced the exposure. For **Paper II, IV and V**, we used case-control studies nested into a prospective cohort to assess the risk of T2D and CVD. This allowed us to assess the exposure *prior* to the outcome, which reduced the possibility of reverse causation [196]. However, there is a possibility that potential pre-clinical forms of the disease may have influenced the exposure levels. Additionally, one study (PFAS associations with BMI in **Paper I**) and several analyses (PFAS associations with proteins, metabolites and blood lipids in **Paper II, Paper IV** and **Paper V**) were cross-sectional, *i.e.* both exposure and outcome were measured at the same time point. Thus, there is no temporal precedence, and the directionality of the associations cannot be guaranteed. It is not likely that BMI or blood lipids influenced PFAS levels as PFAS is not stored in fat tissue and is known to bind to albumin instead of lipids in the blood, but it is possible that certain blood proteins or metabolites influenced PFAS partitioning.

6.3.3 Systematic error: Bias and confounding

Systematic error refers to a systematic deviation of the observed values from the true values. These errors are not related to chance and can skew risk estimates towards, away and across the null, thereby affecting the internal validity of a study. Three major types of systematic error are selection bias, information bias and confounding [197]. They are discussed below and illustrated with the use of directed acyclic graphs (DAGs).

6.3.3.1 Selection bias

Selection bias may arise when there are systematic differences between the sample population (selected individuals) and the population that the sample was selected from

(non-selected individuals) as illustrated in **Figure 17** [196]. This bias can be introduced during recruitment of participants or during the tracing of participants for their outcome status. Bias during recruitment is more common in case-control studies, but not in cohort or nested case-control studies as the cases and controls are selected *prior* to the occurrence of the outcome [198]. However, in this case, loss-of-follow-up and missing data could still introduce a selection bias [196]. Loss-of-follow-up could be an issue in the VIP cohort, as we included only participants that provided blood at both baseline and follow-up. However, a dropout analysis performed in the VIP cohort indicated only a small social selection (**Paper II**) [142]. In contrast, it is unlikely that we have missed many myocardial infarction and stroke cases as the completeness of the Swedish National Patient Register (**Paper IV-V**) is extremely high and more than 99% of hospital discharges are registered [199]. Nevertheless, it is possible that we have missed some cases with minor MI or stroke that were not hospitalized or were outside hospital deaths without autopsy, but this is likely only minor with marginal impact on the results.

Additionally, a particular type of selection bias, which may be of relevance in **Paper III**, is called index event bias (or collider stratification bias). This results from inclusion of subjects based on the occurrence of an index event (first time CHD) and can lead to counter-intuitive results or survival bias [200]. As we studied risk factors for secondary CHD events, our study participants consisted of first event survivors, who could have been different from the non-survivors and conditioning on survival could open a biasing pathway between the exposure and the outcome (**Figure 17**).

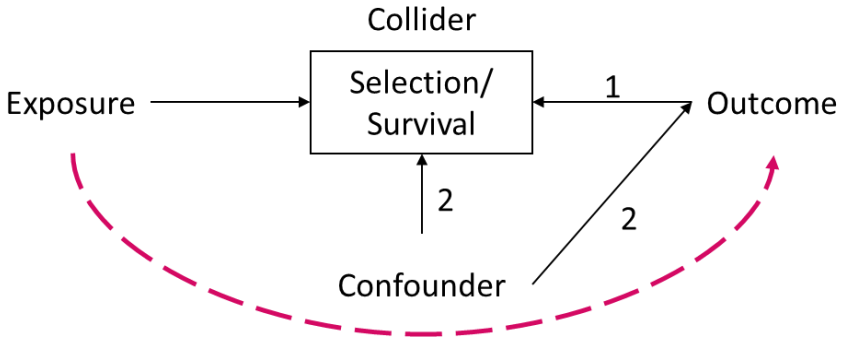


Figure 17. Directed acyclic graph illustration of selection/collider bias. Conditioning (rectangle box) on a collider opens the pathway between the exposure and the outcome directly (option 1) or via common causes of the collider and outcome (confounder, option 2). This creates an association between exposure and outcome, even though they are conditionally independent (unassociated). (Source: Personal collection).

6.3.3.2 Information bias

Information bias may arise due to misclassifications of exposures or outcomes and can be differential (frequency of misclassification is not the same in both exposure/outcome groups) or non-differential (frequency of misclassification is the same in both exposure/outcome groups). Differential misclassifications can cause spurious associations whilst non-differential misclassifications generally, but not always, bias the

estimates towards the null [196]. Examples of differential misclassification are recall bias, *i.e.* cases remember their exposure differently from controls, and diagnostic suspicion bias, *i.e.* exposed subjects have a different likelihood to be diagnosed. In the studies presented in this thesis though, any exposure misclassification is expected to be non-differential as the outcome has not yet occurred at the time of measurement. Likewise, outcome misclassification is expected to be non-differential between exposure categories as subjects are unaware of their exposure levels. The measurement of contaminants in the blood using mass spectrometry decreases the risk of exposure misclassification, as internal levels are measured instead of estimated. Previous studies have indicated that the 10-year intra class correlations were high for both PFAS (0.52-0.85) [41] and OCs (0.5-0.8) [201]. Additionally, although measurement method and sampling tissues were the same within studies, this was not always the case between studies, which makes comparisons of exposure levels more complex (**Paper I**). The use of register data also decreases the risk of outcome misclassification. Validation studies indicated that diagnosis for type 2 diabetes was correct for 95% of the patients (**Paper II**) [143], whilst diagnosis for CVD was correct in 98% for myocardial infarction, 98.6 % for stroke and 68,5% for nonfatal stroke in validation studies in subgroups (**Papers IV-V**) [199]. For **Paper I**, most studies reported BMI measured by nurse/physician as outcome, but a few studies had self-reported BMI which is liable to misclassification. However, exclusion of these studies only marginally impacted the estimates.

6.3.3.3 Confounding

Confounding is a concern when there is a third variable which affects both the exposure and the outcome and is not a result of either, potentially creating or negating an association between the exposure and outcome (**Figure 19**) [196]. In observational studies we can never assume the absence of confounding and instead aim to control for it. However, we are often forced to decide on confounders without full knowledge on the underlying causal structure and this requires some consideration.

Age and sex are considered strong confounders as they have a large impact on both PFAS (accumulates with age and women have lower PFAS levels due to menstruation, breastfeeding and parity) and cardiometabolic disease outcomes (risk increases with age and risks are different for men and women). Therefore, we matched cases and controls based on age and sex (**Paper II, IV-V**) and standardized the BMI for age and sex (**Paper I**). Diet is one of the main sources of exposure for several POPs via consumption of contaminated foods – particularly fish or fast food, whilst diet also impacts risk of cardiometabolic diseases. Therefore, diet could create false associations between PFAS and cardiometabolic diseases and this has been proposed as a reason for the diverging results between different studies [41]. We have adjusted for healthy diet score/fish consumption in our studies, which hardly impacted estimates, but there could be residual confounding. In addition, for the OMICs results, we found associations of PFAS with other

food-related markers and exogenous chemicals, which is likely related to similar sources of exposure or similar partitioning mechanisms.

This potential confounding through excretion or partitioning mechanisms is extremely complex. On the one hand, PFAS excretion in bile is shared with cholesterol [92] and thus, lower bile excretion could potentially increase both PFAS levels and cholesterol. On the other hand, bile excretion could also be on the mediating pathway between PFAS and cholesterol associations (as illustrated in **Figure 4**). Both possibilities would explain PFAS associating with elevated cholesterol and it is very difficult to distinguish them without temporal precedence, which, in turn, is difficult to establish for exposures with long half-lives. However, two findings from our studies suggest that the findings are not due to confounding by bile excretion. First, we might expect higher PFAS levels amongst subjects with specific genetic polymorphisms influencing bile excretion, but we did not find any genetic polymorphisms in the multi-OMICs analysis on the pathway of PFAS-CVD (although this could also be a power issue due to limited sample size; **Paper V**). Second, we might expect that subjects with perturbed bile excretion are already more likely to use cholesterol-lowering medication, as they would be more vulnerable to elevated cholesterol, and when we excluded these subjects, we still found associations between PFAS and cholesterol (**Paper IV**). Another possible confounder is PFAS excretion in urine, as this could be related to kidney function, which is associated with BMI [202]. However, this would not explain our inverse findings and we have no reason to believe that kidney disease should be an issue in our populations (particularly in **Paper I**, where we assessed associations between PFAS and BMI in teenagers). Lastly, for confounding by partitioning mechanisms, PFAS binds to albumin in blood and lower albumin levels could be the result of kidney or liver disease or inflammation and may also be associated with unfavorable metabolic profile [203]. This mechanism could be an explanation for our inverse findings, although it would not explain the association between PFAS and elevated cholesterol.

In **Paper V**, we found that adjustment for age and lipids impacted estimates for associations between OC exposures, OMICs components and cardiovascular outcomes. One limitation in our machine learning approach is that we were not able to adjust for confounders in the random forest modeling. Thus, the first selection step for OMICs features related to POPs may have been biased by confounders. To reduce this impact, we have followed up with confounder adjustment in a second selection step (Spearman correlation). Age may present an important confounder for OCs and the OMICs components, as OC levels increase with age due to accumulated exposures [204] and age is an important determinant of proteins and metabolites. However, age may also be important for the causal relationship by being an important determinant of the exposure and adjustment may take away too much of the variability [189]. Furthermore, higher age groups may also be more vulnerable to pollution damage as they already have accumulated mitochondrial damage. Moreover, it is unclear whether BMI and lipids should

be considered as confounder or mediator in these analyses [157], as they have been found to influence partitioning of OCs between serum and adipose tissue [205], but have also been suggested to be causally linked to OC exposure [206]. In line with this, we found positive associations between a POP-related OMICs component and triglycerides. Another reason to be careful when adjusting for or stratifying by mediators is that this could open another biasing pathway via uncontrolled common causes of PFAS, BMI/lipids and cardiometabolic disease outcomes (collider bias, illustrated in **Figure 17**) [157]. Thus, our observation of stronger associations with lipids in obese subjects, could indicate effect modification by BMI, but could also be collider bias and should be interpreted with caution (**Paper IV**). A particular type of confounding in genetics studies is population stratification and refers to differences in allele frequencies between outcome groups caused by systematic differences in ancestry [207]. It is not likely that this has biased estimates in **Paper III** as most studies had >90% of participants with European ancestry and stratifying studies with >95% of participants with European ancestry *vs* non-European did not change associations for the primary outcome.

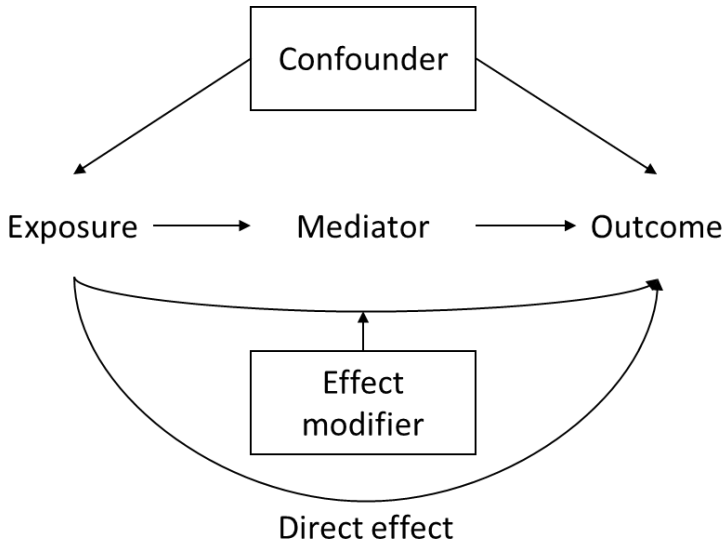


Figure 19. Directed acyclic graph of different types of associations. Confounding indicates a common cause between exposure and outcome and this should be adjusted for (rectangular box). Mediation is a causal pathway and should not be adjusted for. Effect modifiers should be conditioned on (rectangular box), as the effect between exposure and outcome may be different between effect modifier strata. The direct effect indicates conditional dependence (association) between exposure and outcome. Another type of association is collision, which is illustrated in Figure 17 (selection bias can be a form of collider bias) and this should not be adjusted for or conditioned on. (Source: Personal collection)

6.3.4 Random error: Sample size and multiple testing

The random error of a study refers to the precision of the study estimates and is reflected by the confidence intervals [196]. The random error increases with smaller sample sizes and therefore we aimed to increase the study sample sizes by pooling data from different cohorts or by using two controls per case. Multiple testing is another issue that can increase the random error [208]. We have performed many tests but refrained from performing multiple testing adjustment (except for in **Paper II**) for two reasons. First, some exposures under investigation (pollutants or OMICs features) are correlated and therefore not entirely independent, thus multiple testing adjustment may be too stringent. Second, there is still no good way to perform multiple testing adjustment. Current practice often excludes the weaker findings (highest p-values), but it is impossible to know if these were indeed false findings. Instead of doing multiple testing adjustments, we have used other approaches to deal with potential false positives. For OMICs, we used machine learning analysis (random forest) to include all features at once and select the best predictors instead of testing one feature at the time (**Paper II and V**). Nevertheless, this approach together with our limited sample size may have been less suitable for the genetics data in **Paper V**, as we were unable to find polymorphisms linking POP exposures to CVD. For multiple PFAS, we used mixture analysis (quantile G-computation) including all measured PFAS exposures, which reduces the number of tests and deals with collinearity of the exposures (**Paper I**).

6.3.5 External validity

The external validity or generalizability of a study indicates the applicability of the study results to a broader population [196]. The studies used in this thesis have looked at low PFAS exposures, which is valid for most populations [45]. There are however also contaminated areas where people will be exposed to much higher levels, in which case effects could be different. We furthermore restricted some of our studies to specific age ranges (BMI in adolescents, T2D in adults, CVD in adults) and thus results may not be directly applicable to other ages. Additionally, as mentioned before, systematic differences between participants and non-participants (*e.g.* higher education status) may affect generalizability, but the response rates were relatively high for all cohorts (~70%). We have found that there might be effect modifications by sex (**Paper I**) and BMI (**Paper IV**) as well as high correlations with age (**Paper V**), which may indicate that there are vulnerable subgroups (*i.e.* adolescent males, overweight/obese and higher age).

Last, I would like to highlight an important bottleneck in metabolomics studies, which is the large amount of unidentified metabolite features (**Papers II and V**). This makes insight in the underlying mechanisms and comparison between studies harder, which in turn limits generalizability and causal inference.

7 Conclusions

In conclusion, this thesis consistently indicated associations between PFAS and cardiometabolic pathways. PFAS associated with lower BMI in teenagers (**Paper I**), with glycerophospholipids and diacylglycerols that had opposite associations with T2D risk in Swedish adults (**Paper II**) as well as with elevated LDL- and HDL-cholesterol, lower triglycerides and lower risk of myocardial infarction, potentially mediated via lipid and inflammatory pathways (**Paper IV–V**). These results are in line with the current hypothesis of PPARα activation by PFAS, which could impact these pathways and lead to lower cardiometabolic disease risk. Nevertheless, we also found that genetic polymorphisms in the *PPARGC1A* gene, a pivotal player in similar pathways as PPARα, were not associated with secondary cardiovascular disease (**Paper III**), which did not highlight importance of these pathways in disease progression. Additionally, we found that different PFAS compounds do not always follow the same associations (**Paper I**), that PFAS could affect different molecular pathways which may have opposite effects on disease risk (**Paper II**), and that other pollutants from similar sources could have different effects (**Paper IV**).

The findings imply associations of PFAS exposure with metabolism with potential impacts on cardiometabolic disease risks. Yet, as the findings indicate more of an association with lower risk, T2D and CVD may not be regarded as the most relevant outcomes for risk assessments. On the other hand, interference with glucose homeostasis, lipids and myocardial infarction at the exposure concentrations found in the general population, should be perceived as an uncontrolled and undesirable pharmacological intervention on the whole population. This also includes small children and pregnant women and as most processes in the body are connected, there may be other unforeseen adverse effects. Thus, the potential impact of the exposure to PFAS warrants further attention.

8 Points of perspective

This thesis furthermore highlights the importance of mixture and exposome studies and the advantages as well as challenges with the inclusion of multi-OMICs data in epidemiological studies to gain insight in markers and molecular pathways.

Thus, future research should focus on further developing methods and interpretations of exposome and multi-OMICs studies. Additionally, it is important to combine epidemiological study results with laboratory studies to take full advantage of strengths and limitations of both approaches and understand mode of actions underlying associations. Human data is needed to overcome the biological differences between *in vitro/in vivo* studies and humans. Simultaneously, laboratory studies with multiple timepoints and more controlled environments are necessary to understand whether reverse causation and confounding take precedent for the associations between exposures and health outcomes observed in epidemiological studies. These approaches will provide clearer insight in which exposures are most important and for which human health outcomes.

Furthermore, I wish to highlight that chemical pollution of the earth should not only be viewed from the consequences it may have on human health. As not one single study should be seen as the basis for any risk assessment, so should also not a single research field be used as the basis for assessment of planetary health. Planetary health looks at the totality of the natural systems and therefore, studies relating to different animal species and ecosystems should also be considered when assessing chemical risks and solutions.

9 Acknowledgements

I would like to thank and acknowledge everyone who has been with me on this PhD journey. Especially chocolate, dog videos and my yoga-mat. Just joking.

Words can't express how extremely grateful I am for my supervisor Professor Agneta Åkesson. Thank you for your guidance in the field of epidemiology, for always being on my side and believing in me (and encouraging me to do the same), allowing my creative freedom with pink color schemes and above all, for being patient, understanding and kind when I needed it. I couldn't have wished for a better supervisor.

I would also like to thank my co-supervisors, Associate Professors Carl Brunius and Karin Leander and Dr. Carolina Donat-Vargas. Carl, your enthusiasm for science and bubbly personality never failed to inspire me. I so much enjoyed all our discussions, your help with coding and how we are always on the same page. Karin, I really enjoyed working with you and your eye for detail and precision with how to convey meaning through words, has taught me a lot. Carolina, it was so nice to have you as my co-supervisor. I really appreciate how you were always there to help me, with your knowledge or with supportive and encouraging words.

I appreciate my co-authors for the fruitful discussions we have had. In particular, I would like to thank Lin for her guidance in metabolomics and triplots, Kati for her help with metabolite identification and Ingvar for the invaluable advice for additions to improve the manuscripts. Furthermore, I want to thank Rikard for his important feedback on the omics studies, Anton for his help with metabolite identification and, especially, Yan with whom I have had the pleasure of working together a lot on my final paper.

Big thank you also to my unit and colleagues at IMM. Especially, Emilie, for sharing the PhD journey with me and Fredrik, Shuai and Niclas for all lunch and coffee breaks throughout the years and for sharing my love for cookies and cake. Also special thanks to Helen for always inviting me for PFAS events and to Antero, Lars and Lara for all the nice chats. I desperately want to thank Federica, but she hates acknowledgements, so I will just say this: I'm happy to have gotten so close to you during my PhD (quite literally in the office). Big hug to Paolo as well, for being my mentor and a statistics genius that I could always reach out to.

To my friends whom I met in Stockholm: thank you for having made this formerly unknown place a home. Especially Guljan – who encouraged me to stay in Sweden for a PhD and then left for a PhD in the Netherlands, but I'm not bitter about that – and Lotta, I still cherish the days in Lidingö and our dinner parties always feel like family night. Paty and all the wonderful people I have met sharing my excitement for dance, acrobatics, yoga and combinations thereof, thank you for all the warm conversations and fun.

To my Dutch friends: you are all amazing. Aniek, we had so much fun growing up together and I love how we are still non-stop giggling like 5-year-olds whenever we are together. Lieke, we went from best hockey buddies to so many different places in life, but it means a lot to me that we can always talk about everything – for hours. Gonneke and Jorien, I don't know how I would have gotten through these past few years without you. Thanks for making the beautiful moments even more special and for being there with me on the difficult ones too. Spending time with you, sitting on the couch, sipping tea and chatting, has become something I look forward to the most.

Thank you for all the help and support throughout the years to my family (which is rather big, so I will have to refrain from mentioning everyone by name) and to some of our closest family friends: Suzan, Petra, Ingrid, Rudy and Riet.

Last, but not least, I would like to thank Jeremy, my brother Rob and my parents Luc and Ingrid. Jeremy, for being there for me, for always listening to me whining about all the things I worried about in my PhD, not being mad when the same song plays every single evening during my yoga sessions and letting me steal your food when I, once again, didn't have time to buy my own. Rob, we have been through a lot these past years, but we have managed together somehow and I'm proud of our strong bond, strength and perseverance. To mum and dad, you have showed me the meaning of life. Your love and encouragement – to do my best but never forget to enjoy life – has paved the way for me to be where I am today. From dad's valiant attempts to teach me patience in the path to learning and mom's warm kindness always reminding me that it's okay to fail too. Wish you were here to see this achievement. Thank you for everything.

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