Master Thesis

Effect of delayed processing and freezing on selected components in EDTA-plasma

Submitted by

Liv Paltiel

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Univ. Prof. Berthold Huppertz, PhD

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Statutory Declaration

I declare on my honor that I have written this dissertation independently and without assistance, that no sources other than those cited were used and that the sources used verbatim or in substance have been marked as such.

Graz, June 30th,2022 *Liv Paltiel. eh*

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Abstract - deutsch

Hintergrund

Für Biobanken ist es von größter Bedeutung, den Wissenschaftlern biologisches Material von hoher Qualität zur Verfügung zu stellen, das für den jeweiligen Zweck geeignet ist und somit reproduzierbare Ergebnisse liefern kann. Um dies zu erreichen, muss die Probenqualität während der gesamten Lebensdauer der Bioproben aufrechterhalten werden. In diesem Projekt untersuchte ich, wie spezifische Analyte in EDTA-Plasma durch verzögerte Zentrifugation von Blut und verzögertes Einfrieren von Plasma beeinflusst werden.

Methoden

Um die Stabilität von Natrium, Cholesterin, Triglyceriden und 23 verschiedenen Perfluoralkyl- und Polyfluoralkylsubstanzen (PFAS) in EDTA-Plasma nach dem verzögerten Einfrieren zu untersuchen, wurden die Plasmaproben nach der Trennung vor dem Einfrieren 0, 4, 24, 48 und 72 Stunden bei Raumtemperatur gelagert. Außerdem wurden die Blutproben vor dem Zentrifugieren 0,5, 4, 24, 48 und 72 Stunden bei Raumtemperatur gelagert, um die Stabilität nach der verzögerten Verarbeitung zu untersuchen. In jedem der beiden Teile des Projekts wurde 24 Teilnehmern Blut abgenommen.

Ergebnisse

Die Untersuchung der Auswirkungen des verzögerten Einfrierens von Plasma zeigte keine statistisch signifikanten Unterschiede bei Cholesterin und den meisten der 11 PFAS, die bei einem oder mehreren Teilnehmern nachgewiesen wurden. Statistisch signifikante Unterschiede wurden bei den Konzentrationen von Natrium, Triglyceriden, PFNA und PFTrDA auf Gruppenebene im Laufe der Zeit festgestellt. Die verzögerte Zentrifugation des Blutes führte zu statistisch signifikanten Unterschieden zwischen den Zeitpunkten für Natrium-, Cholesterin- und PFDA-Konzentrationen.

Schlussfolgerung

Diese Studie hat gezeigt, dass eine verzögerte Verarbeitung von Biobankproben bis zu 72 Stunden die Konzentrationen einiger Analyten beeinflusst, während andere nur geringfügige oder keine Veränderungen aufweisen. Daher wird die Notwendigkeit einer Dokumentation der verzögerten Verarbeitung von Proben und einer Validierung der Probenbehandlung für die Analyse spezifischer Analyten unterstützt.

Abstract

Background

For biobanks, it is of utmost importance to provide scientists with biological material of high quality that is fit for purpose and thus can generate reproducible results. To achieve this, sample quality must be maintained throughout the lifetime of the biospecimens. In this project we are evaluating how specific analytes in EDTA-plasma is affected by delayed centrifugation of blood and delayed freezing of plasma.

Methods

To investigate the stability of sodium, cholesterol, triglycerides and 23 different perfluoroalkyl and polyfluoroalkyl substances (PFAS) in EDTA-plasma after delayed freezing, plasma samples were stored at room temperature for 0, 4, 24, 48 and 72 h after separation before freezing. In addition, blood samples were stored at room temperature for 0.5, 4, 24, 48 and 72 h before centrifugation to investigate the stability after delayed processing. Blood was collected from 24 participants in each of the two parts of the project.

Results

The study of the effect of delayed freezing of plasma demonstrated no statistically significant differences in cholesterol, and most of the 11 PFAS that were detected in one or more participants. Statistically significant differences were shown in sodium, triglycerides, PFNA and PFTrDA concentrations on group level over time. Delayed centrifugation of blood resulted in statistically significant differences between timepoints for sodium, cholesterol and PFDA concentrations.

Conclusion

This study demonstrated that delayed processing of biobank samples up to 72 h does affect the levels of some analytes, while others only have minor or no changes. Hence, the need for documentation of delayed processing of samples and validation of sample handling for analysis of specific analytes are supported.

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Glossary und Abbreviations

6:2 Cl-PFESA	9-chlorohexadecafluoro-3-oxanonane-1- sulfonate
8:2 Cl-PFESA	11-chlorohexadecafluoro-3-oxanonane-1- sulfonate
ADONA	Ammonium salt of 4,8-dioxa-3H-perfluorononanoate
ANOVA	Analysis of variance
BRISQ	Biospecimen Reporting for Improved Study Quality
CEN	European Committee for Standardization
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine-Tetra-acetic Acid
GP	General Practitioner
HDL	High-density lipoprotein
HFPO-DA	Ammonium salt of 2,3,3,3,-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-propanoate
ISBER	International Society for Biological and Environmental Repositories
ISO	International Organization for Standardization
LC	Liquid chromatography
LDL	Low-density lipoprotein
LOQ	Limit of quantification
MoBa	The Mother, Father and Child Cohort Study
MS	Mass spectrometer
NIPH	The Norwegian Institute of Public Health
PFAS	Per- and polyfluoroalkyl substances
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
OECD	Organization for Economic Co-operation and Development
REC	Research Ethics Committee
RNA	Ribonucleic acid
SPREC	Standard Preanalytical Code
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization

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1 Introduction

1.1 The biobank of the Norwegian Institute of Public Health

In Norwegian law and regulations, the definition of a research biobank is a collection of human biological material that is used in a research project or that is going to be used for research (1). Department of biobanks at the Norwegian Institute of Public Health (NIPH) manages several research biobanks in different projects – mainly in health surveys and biobanks for epidemiological research.

1.1.1 The Norwegian Mother, Father and Child Cohort Study

The Norwegian Mother, Father and Child Cohort Study (MoBa) is a prospective population-based pregnancy cohort study which includes almost 113.000 pregnancies. The aim is to find causes of diseases among the children and parents (2-4).

From June 1999 to the end of 2008, pregnant women were recruited to participate in the study, and they consented for themselves and for the child(ren) in their wombs. From 2000, also their spouses were invited (2,3,5,6) The families participate with biological material and questionnaires, and MoBa follows the participants with a lifelong perspective (7). In total, MoBa includes more than 95 000 mothers from whom many participate with more than one pregnancy, 114 000 children and 75 000 fathers (4).

In MoBa, biological material was collected in 50 different hospitals and by some private gynecologists. The samples were collected from both parents at the time of ultrasound at 17-18 weeks of gestation and from the mother and the umbilical cord after birth (5,6). The samples were shipped to the central biobank at the Norwegian Institute of Public Health (NIPH) by ordinary mail, express packages or by car from the closest hospitals. There were neither cooling of the biological material during shipment nor monitoring of temperature in the shipments. Approximately 3 % of the samples arrived at the central biobank the collection day, 54% the following day and 14 %, 18 % and 7% after 2, 3 and 4 days respectively (6).

The set of samples collected varied during the collection period and has been described by Rønningen et al. (5) and Paltiel et al. (6). The final sample set available from the mothers

collected at 17-18 weeks of gestation consists of whole blood (EDTA), urine, DNA and EDTA-plasma where one sample was centrifuged and plasma transferred at the time of collection whereas one sample was centrifuged and plasma transferred after arrival to the central biobank. For the children, the final sample set includes whole blood (EDTA), DNA, blood in a Tempus RNA preservation tube and EDTA-plasma which was centrifuged and transferred to the biobank after shipment. The sample set collected from the fathers and from the mothers after birth consists of whole blood (EDTA), DNA and EDTA-plasma which was centrifuged in the hospitals before shipment (5,6).

1.1.2 The Norwegian Human Environmental Biobank

To gain more knowledge about body levels of environmentally hazardous chemicals in Norwegians and their impact on health, a biobank has been established by the Norwegian Institute of Public Health and blood and urine samples have been collected for research and for monitoring environmental contaminants in the population. The Norwegian human environmental biobank is a substudy of MoBa and will be a resource to assess environmental contaminants, including changes over time, identify population groups at higher risk, assess exposure pathways, among others (8).

To date, the Norwegian human environmental biobank consists of two parts and a third part is being planned. Part I consist of retrieval and analysis of whole blood, plasma and urine samples from 2999 pregnant women in the Norwegian Mother, Father and Child Cohort Study. For these women, sampling during pregnancies was done in 2002-2008 (9).

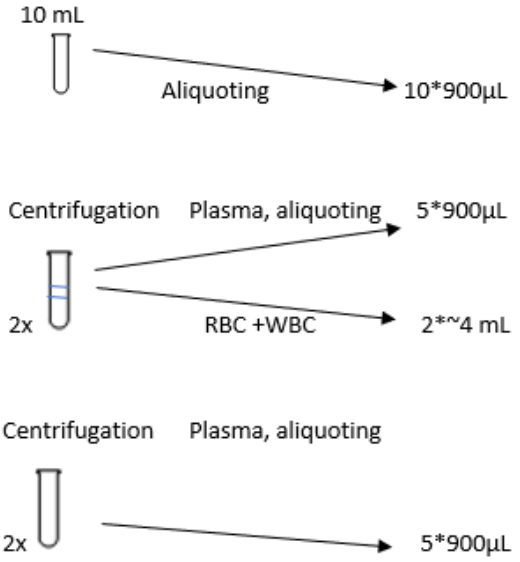
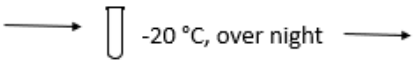
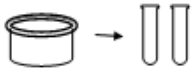
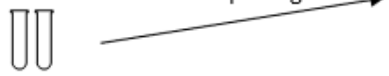
Part II was conducted in 2016 when the 2999 participating women in part I were invited to participate together with the child from the specific pregnancy in part I and the child's father. This part consists a of a new collection of biological material from all the participants and a questionnaire regarding lifestyle and diet for the adults (8,9). A total of 658 mothers, 500 fathers and 668 children have participated with biological material in part II of the study.

In this study, samples were drawn in the participant's GP offices all around the country. Blood samples collected in the doctor's office for the adults were five 10mL EDTA- tubes with whole blood, one 10 mL tube without anticoagulant and one 3 ml Tempus tube for RNA preservation. From the children, blood was collected in four 5 mL EDTA-tubes, one

5 ml tube without anticoagulant and one 3 ml Tempus tube for RNA preservation. In addition, 16 ml of urine in boric acid tubes were collected from all participants.

Centrifugation of plasma and serum samples were conducted after arrival in the NIPH biobank. Table 1 shows the collected specimens at all stages during collection and processing in the biobank.

Table 1: Flow chart for sample collection and handling in Human Environmental Biobank Norway – samples from mothers and fathers

Samples collected	**Processing at doctor's office, before shipment by ordinary mail**	**Handling in the biobank, NIPH**	**Storage, -80 °C, NIPH**
5*10-mL EDTA blood 	No processing		 Whole blood in 2D barcoded tubes  EDTA-plasma in 2D barcoded tubes  RBC +WBC in original tube  EDTA-plasma in 2D barcoded tubes
10 mL blood without anticoagulant 	No processing	Centrifugation Serum, aliquoting 5*900µL 	 Serum in 2D barcoded tubes
3 mL blood in 9 mL Tempus tube for RNA 	No processing		 Tempus tube
Urine 	Transferred to two 10 mL tubes with boric acid 	2x10 mL Aliquoting 10*900µL 	 Urine in 2D barcoded tubes

Like MoBa, in the Norwegian Human Environmental Biobank the samples were sent from the general physicians to the central biobank by ordinary mail without any cooling or monitoring of temperature. In table 2, time from blood draw until the samples arrived in the biobank is presented for MoBa and the Norwegian Human Environmental Biobank.

Table 2: Time for collection till the samples are received in the NIPH biobank 1999-2016

	Same day	1 day	2 days	3 days	4 days	5+ days
Human Environmental Biobank	7 (0 %)	1 232 (67 %)	281 (15 %)	180 (10 %)	89 (5 %)	50 (3 %)
MoBa	11 767 (3 %)	191 293 (54 %)	51 029 (14 %)	62 515 (18 %)	25 522 (7 %)	12 105 (3 %)

In 2022, collection of biological material in part III of the Norwegian Human Environmental Biobank is planned. Also for this collection, it is planned to use ordinary mail for shipment.

However, in 2018 the shipment conditions for ordinary mail in Norway changed. Time from shipment to reception for ordinary mail has gone from one to two working days. In another collection the NIPH biobank manages, urine samples are collected at home and sent from the nearest Post office or Post in Shop by ordinary mail. In the beginning of the study, before the change, 26 % and 25 % of the samples arrived at the biobank one and two days after sample collection. After the change, less than 1 % of the samples arrive after one day and 11 % after 2 days.

1.2 Sample stability and factors affecting the results of component measurement

Human biological material is collected in biobanks worldwide to help provide answers to important questions about health. The value of the biobanks depends on their ability to provide good and accurate answers and therefore it is of utmost importance that the quality of the biological material is sufficient, and the samples are fit for purpose.

Biological material such as blood and urine that comes from the human body maintains a given temperature inside the body. This is a temperature where components in, for example, plasma and urine will have the best conditions for existence, therefore it is important that a blood or urine sample is treated in such a way that we can recreate the snapshot from when the sample was taken.

There are various factors that affect the results of measurements of sample analytes. In addition to the biological factors, these factors are found in the preanalytical, analytical and postanalytical phases. Several studies have reported that the majority of measurement errors, up to 68,2 % is caused by failures from the pre-analytical phase followed by the post-analytical and analytical phases (10-13). These publications come from clinical laboratories, but this will also affect biobanking and science and thus the possibility to drive the medical, biomedical and other fields forward. Knowing that there is a replication crisis in science with an increasing number of retractions of publications and a significant amount of biomarker research which cannot be replicated (14,15), the focus on standardized procedures and procedures which gives high quality biological material is of utmost importance.

1.2.1 The pre-analytical phase

In biobanking, the preanalytical phase consists of all sample handling from sample collection until the component analysis begins. To provide valid results in analytical testing of biological samples, the level of the sample component should not change from sample collection (or from their natural state in the body) until the performance of the analysis. Both in clinical settings and in biobanks, this can be difficult. Therefore, it is important to have knowledge of how the processing of samples after sampling, via freezing and long-term storage in a biobank can affect the level of components in the samples, as well as documentation of the sample handling throughout the life cycle of the biological material (16,17).

Nevertheless, the work in a biobank is pre-analytical or mostly pre-analytical and all sample handling can affect the results when analyzing the samples. Several factors may affect the samples and subsequently the sample quality and in figure 1 examples of pre-analytical factors that may affect sample quality in a biobank are presented.

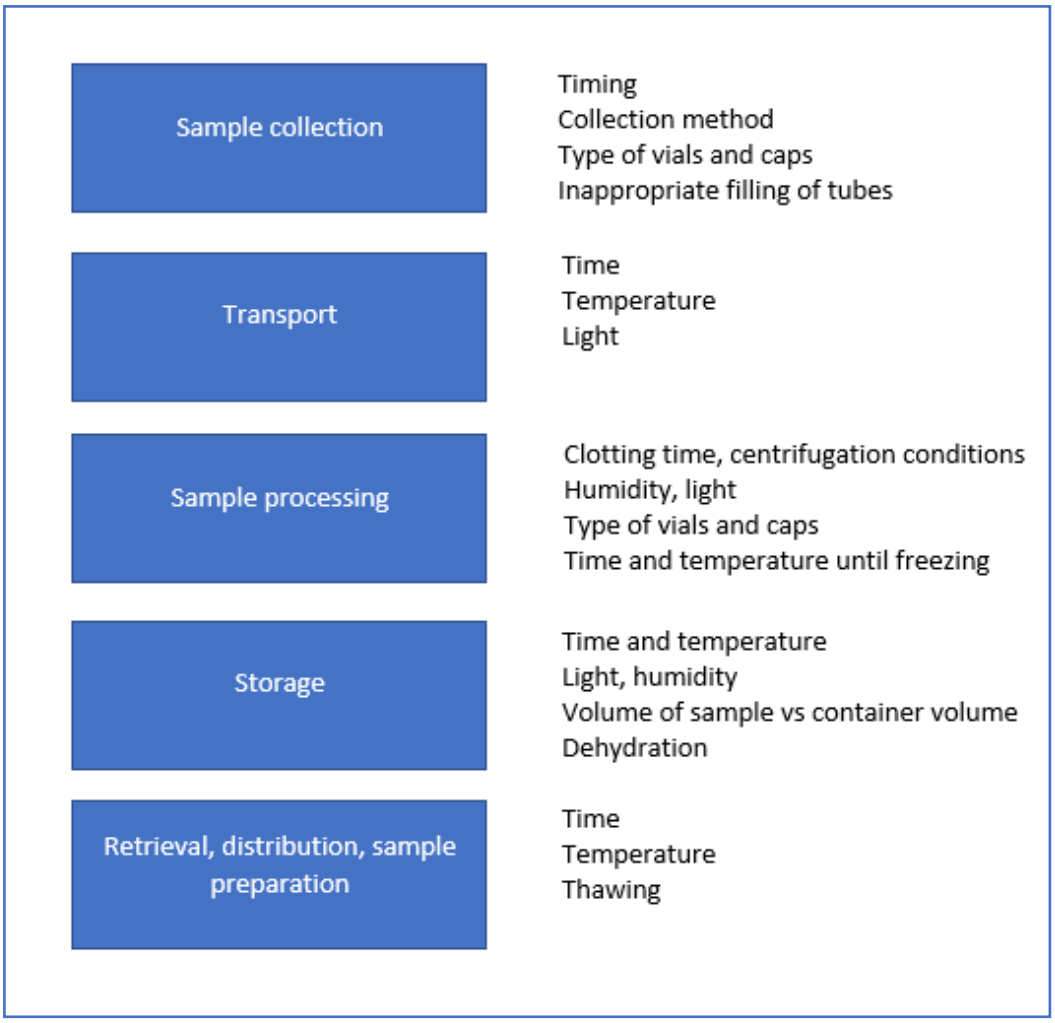


Figure 1: Examples of pre-analytical factors in a biobank

1.2.2 Stability of plasma-EDTA samples

1.2.2.1 Pre-analytical handling

As shown in figure 1, there are several factors that can affect sample quality and thus the result of biomarker analysis. For plasma, both pre- and post-centrifugation storage conditions are important for sample quality (18).

To obtain plasma, the recommended procedure for centrifugation of whole blood is within 0.5 to 2 hours after the blood is drawn (19). Even though rapid centrifugation is essential for some analytes, and delayed centrifugation can cause leakage of compounds from the red cells to the plasma (18), others have been found to be more stable and might be stored at room temperature up to one week (20).

In biobanking, the focus when it comes to delayed processing has often been on delayed centrifugation and few studies have investigated delayed analysis or delayed freezing of the plasma after separation of plasma from blood (18).

1.2.2.2 Compounds

1.2.2.2.1 Sodium

Sodium is one of the mayor intracellular cations and helps the body to maintain the fluid balance and regulation of blood pressure. It is also needed for muscle and nerve functions (21-23). Thus, sodium is one of the most often assayed electrolytes in clinical settings (21). The source of sodium for humans is through salt, sodium chloride, in food where approx. 40 % is sodium and 60 % chloride (23-24). In Norway, the diet recommendations for adults suggest that the body needs about 0.6 g sodium daily to function, which is approx. 1.5 g salt, and recommended maximum intake is 5 g salt per day (24). Health effects that can occur with high intake of sodium are high blood pressure which can contribute to stroke, heart attack, eye changes and kidney damage (24).

Sodium is an element and cannot degrade. Therefore, changes in plasma concentrations will have other reasons like volume changes or effects on other components in plasma like lipids, nucleic acids or proteins which bind or release sodium in the liquid phase. According to Taylor et al, sodium is stable at ambient temperature for 5 days (18) and the product sheet for the analysis of sodium used in this project suggests that sodium is stable in plasma for 14 days at 15-25 °C (21). When it comes to storage of whole blood samples before separation of plasma or serum, Hedayati et al. have reported that a delay of 56 hours at room temperature provides reliable results for serum samples (25).

1.2.2.2.2 Cholesterol

Cholesterol is a lipid, which consist of a steroid skeleton and has a secondary hydroxyl group in position C3 (26-27). In the body, cholesterol is found in cell membranes, and it is necessary for formation of hormones, bile acid and vitamin D. As a result of its non-solubility in water and water-like fluids, it is transported in the blood bound to lipoproteins (26-28). Cholesterol is synthesized in many cells, especially in the liver and intestinal wall, and this covers the need of the human body. In addition, we get cholesterol from the diet, approx. ¼ of the total amount (26-28).

The effect of delayed centrifugation on cholesterol in plasma and serum samples has been investigated by several scientists as reviewed by Hedayati et al. (25). Among them are Clark et al. who have shown that cholesterol concentrations in plasma-EDTA changed by less than 4% after 7 days of delayed centrifugation of whole blood (20). Most studies suggest that whole blood can be stored at room temperature up to one week before centrifugation and separation of plasma without significant change in total cholesterol concentration. However, even if the total cholesterol levels do not change significantly, the subforms high-density lipoprotein (HDL) and low-density lipoprotein (LDL) may behave differently. Clark et al have reported that HDL levels in plasma decrease and LDL levels increase when stored for 7 days.

When it comes to delayed freezing in biobanks or delayed analysis in a biochemistry laboratory, the product sheet for the cholesterol analysis method in this project suggests that the shelf life of plasma samples stored at 15-25 °C is 7 days based on the WHO publication on use of anticoagulants in diagnostics laboratory investigations (29) and Clinical Guide to Laboratory Tests, 3 edition (30).

1.2.2.2.3 Triglycerides

Triglycerides are lipids and they are built up by glycerol and three, usually different, fatty acids which are dependent on the origin. Triglycerides are used as a source of energy and we get them from different types of food, e.g. fish, eggs, milk, meat, cooking oils, etc. High triglyceride levels increase the risk of getting heart diseases (31-33).

Taylor et al. have presented that triglycerides in plasma are stable up to 7 days at room temperature (18). However, the stability of triglycerides in plasma is suggested to be 2 days at 15-25 °C by the producer of the analytical method used for quantification of triglycerides in this project (31).

The effect of delayed centrifugation on triglycerides concentrations in plasma and serum have been investigated on several occasions. Hedayati et al. have presented that 17 of 20 articles in their review have studied the stability of triglycerides at different conditions and that whole blood samples can be stored at least 7 days before centrifugation and plasma separation (25).

1.2.2.2.4 PFAS

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) is a large group of more than 7000 synthetic fluorinated compounds (34). They consist of a carbon skeleton to which fluor atoms are attached (34-35), see figure 2 and 3 for examples of structures. Due to their ability to repel water, stain, and grease, PFAS have been used in numerous of daily products like non-stick cookware, furniture, paint, clothes and food packaging since the 1950s. The main exposure routes for humans are through food, drinking water, dust particles and indoor air (34-36). Infants are exposed through breast milk and the fetus through the umbilical cord (34).

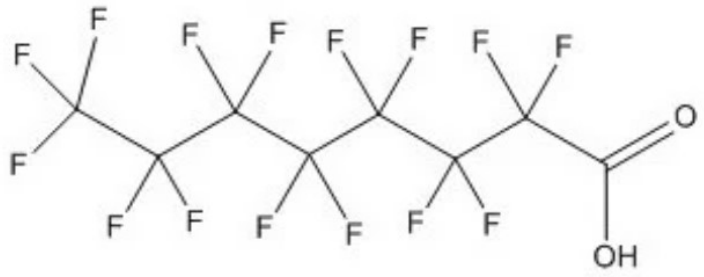


Figure 2: Structural formula for PFOA. Illustration by NIPH.

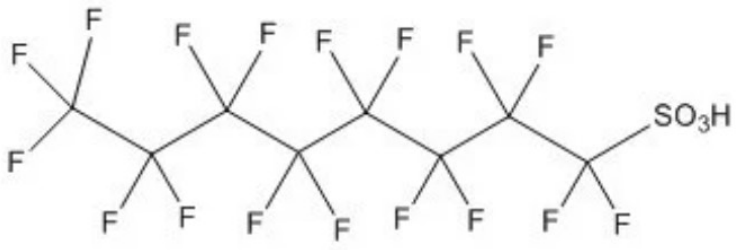


Figure 3: Structural formula for PFOS. Illustration by NIPH.

There is an increasing concern about PFAS and their impact on human and animal health (37), and therefore it is interesting to monitor PFAS concentrations in the humans over time. Effects on health have been studied both in human health and animal studies. Not all effects found in animal studies are relevant for humans and the PFAS levels in these studies are often significantly higher than the relevant exposure for humans. When it comes to the epidemiological studies, many effects studied in human studies do not have enough power to conclude from and epidemiological studies are mainly reporting

associations and not causality. Of all the existing PFAS, the effects of PFOS and PFOA are most frequently studied. (34).

Among the health effects that has been linked to PFAS are decrease in children's birth weight, liver enzyme changes, elevated cholesterol level, hormone disruption, increased risk of cancer and disturbances in the immune system (34, 37, 38). It has been shown that PFOS and PFOA affect the immune system and the effect that is seen with the lowest exposure, is inhibition of antibody development after vaccination (34).

Few studies have investigated whether preanalytical factors lead to changes in concentrations of environmental pollutants like PFAS in plasma samples. Several of the PFAS are persistent and do not degrade in the environment or in humans and some have half-lives of 2-5 years in the environment and in humans (38-39). Other PFAS may be non-persistent and degrade to persistent substances such as PFOS and PFOA. These compounds are often called PFAS precursors. Thus, non-persistent PFAS might be converted in the body, contributing to the concentrations of persistent PFAS in the blood.

1.3 Standardization – standards, guidelines and best practices

As biobanking has evolved during the last decades, the focus on harmonization and standardization of sample collection and handling has become more and more prominent to ensure samples of high quality which are fit for the intended purpose. This can also be seen in the guidelines, best practices and standards that are developed for biobanking.

In 2009, ISBER launched a standard preanalytical code, SPREC, which "*identifies and records the main pre-analytical factors that may have impact on the integrity of sampled clinical fluids and solid biospecimens and their simple derivatives during collection, processing and storage".* (16,40). The second edition came in 2012 and the focus for SPREC is on documenting the different factors that can affect sample integrity like collection tube, centrifugation conditions, storage conditions and several others. SPREC is a great tool for biobanks to identify which pre-analytical factors a biobank should document but also valuable in providing samples fit for the intended purpose and when using samples from different biobanks (40).

Several organizations have developed best practices. Among these are OECD Best Practice Guidelines for Biological Resource Centers (41), National Cancer Institute Best Practices for Biospecimen Resources (42) and Best Practices: Recommendations for Repositories from The International Society for Biological and Environmental Repositories (ISBER) (43) and they all identify sample quality as an important aspect and have recommendations to ensure high-quality samples. As it is said in the introduction of the ISBER Best Practices: "*The availability of high-quality biological and environmental specimens for research purposes requires the development of standardized methods for collection, handling, storage, retrieval, and distribution*" (43).

In addition, guidelines for reporting human biospecimen data in publications, Biospecimen Reporting for Improved Study Quality (BRISQ), has been developed and consists of a list of elements that can influence sample quality. The idea has been to supply others with thorough and standardized data for improved evaluation and comparison of experimental results and thereby a framework to increase the possibility to reproduce results (44).

Until 2018, no international standards for biobanks existed. If biobanks wanted to have a third party put a quality mark on them, they implemented systems and applied for certification after ISO 9001 Quality management systems or accreditation after ISO 17025 General requirements for the competence of testing and calibration laboratories or ISO 15189 Medical laboratories — Requirements for quality and competence. Even if all of these include important issues for a biobank, none of them fully cover the detailed needs of a biobank. Fortunately, in 2018 the International Standard Organization (ISO) launched ISO 20387:2018 Biotechnology – Biobanking – General requirements for biobanking. The document "*specifies general requirements for the competence, impartiality and consistent operation of biobanks including quality control requirements to ensure biological material and data collections of appropriate quality"* (45).

A main focus in the standard is to have biological samples (and associated data) which is fit for the intended purpose. In the standard, the definition of fitness for the intended purpose is "*in line with prearranged requirements for an intended use".* To ensure that samples are fit for the intended purpose, the standard have requirements, e.g. for validation of processing methods and quality controls to "*demonstrate fitness for the intended purpose*".

In addition, a series of standards for pre-examination processes for different biological materials has been launched and more are expected to come. First as CEN technical standards and lately as ISO standards. These ISO standards are based on scientific knowledge and consist of requirements for handling and documentation of samples before and in the laboratories.

1.4 Project description and hypothesis

Considering the transport times from sample collection and to reception in the central biobank in most collections in the Biobank of the Norwegian Institute of Health, two questions has been raised:

1) What effect does delayed separation of EDTA-plasma from blood have on plasma component in samples stored in biobanks?
2) What effect does delayed freezing of EDTA-plasma have on components in samples stored in biobanks?

In this project we are evaluating how EDTA-plasma is affected due to long transport times. A few analytes interesting for The Mother, Father and Child Cohort Study and The Norwegian Human Environmental Biobank have been selected.

The purpose of this study is divided into two parts. One is to assess whether and how time from collection of whole blood in tubes with EDTA as anticoagulant until separation of plasma up to 72 hours later will affect levels of selected analytes. The second is to assess whether and how time from separation of EDTA-plasma until freezing of the samples in -80 °C will affect levels of selected analytes.

2 Methods

2.1 Specimen collection and handling

All samples were collected from adult employees at the Norwegian Institute of Public Health and informed consent describing the project and personal protection and privacy in the project was obtained before sampling.

According to REC, the study is outside the Norwegian Health Research Act, therefore they have declined to evaluate the study. Nevertheless, the principal investigator is responsible for the personal protection and privacy in the project.

2.1.1 Delayed freezing of EDTA-plasma

30 mL of venous blood was collected from each of 24 non-fasting male and female participants, approx. 25-65 years old, from whom 17% were male, in three 10 mL Vacutainer tubes containing EDTA (Becton-Dickinson (BD), Plymouth, UK). The samples were allowed to stand 30 min in room temperature, 20-21.5°C, followed by centrifugation, 1800 x g for 15 min. Plasma was transferred and aliquoted into five 600µL aliquots in Cobas Modular Standard False Bottom Tube (Roche, Basel Switzerland), five 200µL aliquots in 0.5 mL Matrix tubes (Thermo Electron LED, Langenselbold, Germany) and five 900 µL aliquots in 1,4 mL Matrix tubes (Thermo Electron LED, Langenselbold, Germany) from each patient. One aliquot of each size was frozen immediately at -80°C. The other tubes were allowed to stand in room temperature, 20-21.5°C, for 4, 24, 48 and 72 hours, before they were frozen at -80°C, see figure 4.

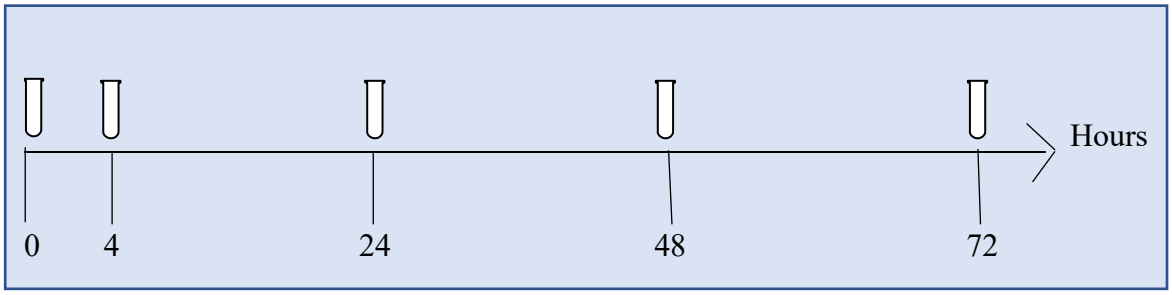


Figure 4: Time from end of centrifugation and pipetting of plasma until freezing of the plasma-EDTA aliquots at -80°C

2.1.2 Delayed separation of blood

30 mL of venous blood was collected from each of 24 non-fasting male and female participants in five 6 mL Vacutainer tubes containing EDTA (Becton-Dickinson (BD), Plymouth, UK). The participants were 25-65 years old and 29% were male. Eight persons participated in both parts of the project. All samples were standing at room temperature, 20-21.5°C, until centrifugation. After 30 min, 4, 24, 48 and 72 h, one EDTA-tube from each participant was centrifuged at 1800 x g for 15 min, and plasma transferred and aliquoted into two aliquots with 200 µL in a 0.5 mL Matrix tube (Thermo Electron LED, Langenselbold, Germany), 900 µL Matrix tube (Thermo Electron LED, Langenselbold, Germany) and 600 µL into a Cobas Modular Standard False Bottom Tube (Roche, Basel Switzerland), see figure 5. All samples were frozen at -80°C immediately after the aliquotation.

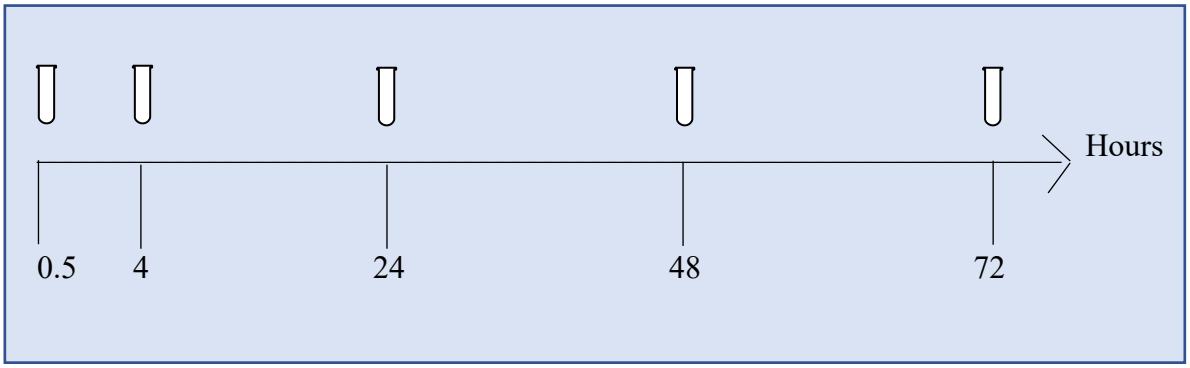


Figure 5: Time from blood collection until centrifugation of whole blood.

2.2 Biomarker analysis

The samples in both parts of the project were analyzed for sodium, cholesterol, triglycerides and 23 different perfluoroalkyl and polyfluoroalkyl substances (PFAS).

Analysis of sodium, cholesterol and triglycerides was performed at the Department of Medical Biochemistry at Oslo University Hospital. The samples were thawed at room temperature and then analyzed. Sodium was analyzed using an indirect ion-selective electrode instrument (Cobas, Roche) (21) and cholesterol and triglycerides were analyzed using enzymatic colorimetric methods (Cobas, Roche) (26,31). The analyses are ISO 15189 accredited, and reference values and detection limits are presented in table 3.

After shipping to the laboratory, a few samples were lost and had to be shipped again. The 900µL aliquot, which was an extra aliquot for possible further analyses, was thawed at room temperature and 0,6 mL was transferred to a 600 µL Cobas Modular Standard False Bottom Tube (Roche, Basel Switzerland) before shipment. Therefore, these samples have gone through one additional freeze-thaw cycle and were not analyzed together with the other samples from the participants. The samples from part one of the project are ID 14, 24 h, ID 21, 72 h and from part two ID 13 at 24 h, ID 16 at 24 h and ID 24 at 48 and 72 h.

Table 3: Analyses of sodium, cholesterol and triglycerides. Reference values, measurement ranges and coefficient of variations.

Compound	Reference value in plasma, adults (mmol/L)	Measurement range (mmol/L)	Coefficient of variation, %
Sodium	137-144	80-180	1.5%
Cholesterol	2.9-6.1 (18-29 years) 3.3-6.9 (30-49 years) 3.9-7.8 (≥50 years)	0.1-20.7	3%
Triglycerides	0.5 – 2.6	0.1-10.0	4%

Analysis of the 23 different PFAS was performed at the Department of Food Safety at the Norwegian Institute of Public Health using 150 µL plasma. The full list of PFAS and limits of quantification (LOQ) are presented in table 4.

The method used is based on column switching liquid chromatography (LC) coupled to a triple quadruple mass spectrometer (MS) as described by Haug et al. (36). In the publication, analysis of 6:2 Cl-PFESA, 8:2 Cl-PFESA, ADONA and HFPO-DA are not described, but they have been analyzed with the same method and simultaneously with the rest of the PFAS.

Table 4: PFAS analyzed with abbreviations and limits of quantification.

Abbreviation	Compound	LOQ (ng/mL)
PFBA	Perfluorobutanoate	0.10
PFPeA	Perfluoropentanoate	0.05
PFHxA	Perfluorohexanoate	0.05
PFHpA	Perfluoroheptanoate	0.05
PFOA	Perfluorooctanoate	0.05
PFNA	Perfluorononanoate	0.05
PFDA	Perfluorodecanoate	0.05
PFUnDA	Perfluoroundecanoate	0.05
PFDoDa	Perfluorododecanoate	0.05
PFTrDa	Perfluorotridecanoate	0.05
PFTeDa	Perfluorotetradecanoate	0.20
PFBS	Perfluorobutane sulfonate	0.05
PFHxS	Perfluorohexane sulfonate	0.05
PFHpS	Perfluoroheptane sulfonate	0.05
PFOS	Perfluorooctane sulfonate	0.05
PFDS	Perfluorodecane sulfonate	0.20
PFOSA	Perfluorooctane sulfonamide	0.05
MeFOSA	N-Methylperfluorooctane sulfonamide	0.05
EtFOSA	N-Ethylperfluorooctane sulfonamide	0.05
HFPO-DA (GenX)	Ammonium salt of 2,3,3,3,-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-propanoate	0.20
ADONA	Ammonium salt of 4,8-dioxa-3H-perfluorononanoate	0.05
6:2 Cl-PFESA	9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	0.01
8:2 Cl-PFESA	11-chlorohexadecafluoro-3-oxanonane-1-sulfonate	0.05

2.3 Statistical analysis

SigmaPlot version 14.0 and SPSS version 27 was used for the statistical analyses. To test if the data follows a normal distribution, Shapiro-Wilks's test was used, and Brown-Forsythe was used to test if the timepoints had equal variance. Since the sample from each participant is measured at each of the five timepoints, Oneway repeated measures ANOVA was used to compare the different timepoints for data that was following a normal distribution. If the difference in mean values between the different times were statistically significant, Bonferroni t-test was used to compare two and two groups. For data that did not follow a normal distribution, a non-parametric Friedman test (Repeated measures ANOVA on ranks) was conducted. To compare two and two timepoints, post-hoc analysis

with Wilcoxon signed-rank test was performed with a Bonferroni correction to adjust for multiple testing. Statistically significance was accepted at $p<0.05$.

3 Results

All samples were analyzed for sodium, cholesterol, triglycerides and 23 different PFAS. The samples from each participant in each part of the project were analyzed in the same runs except for the samples that had to be re-sent. Visible hemolysis was not detected in the samples.

3.1 Delayed freezing of plasma

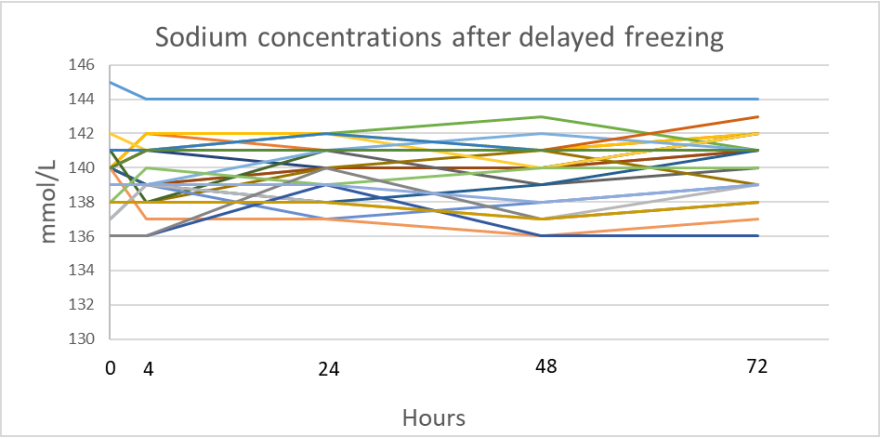
3.1.1 Sodium, cholesterol and triglycerides

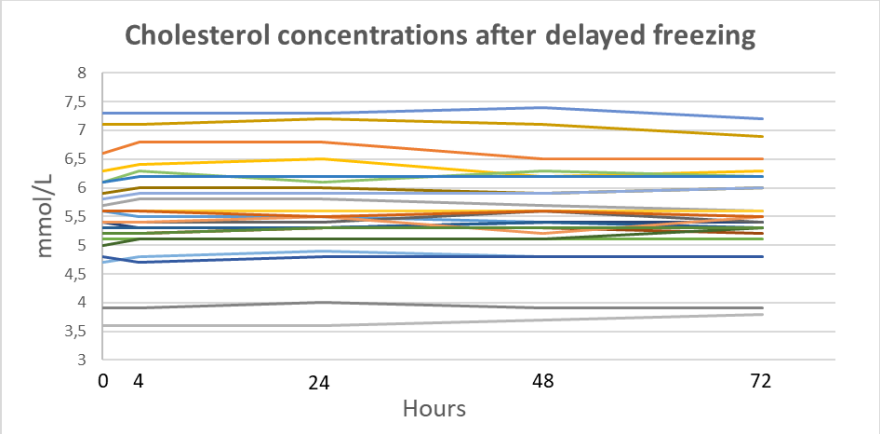
The measured concentrations for all measured analytes in all samples after delayed freezing of EDTA-plasma are found in Appendix 1. For sodium, cholesterol and triglycerides, the samples from ID 14 at 24 h and ID 21 at 72 h went through one additional freeze-thaw cycle before analysis and were analyzed at different timepoints than the other samples from the participant.

Visualization of the measured concentrations for sodium, cholesterol and triglycerides is found in figure 6 a, b and c, respectively. The descriptive values, i.e. mean concentration, standard deviation, median, 25 and 75% percentiles (Q1 and Q3) in addition to the range, minimum and maximum concentrations, for 0, 4, 24, 48 and 72 h are presented in table 5 for sodium, table 6 for cholesterol and table 7 for triglycerides. The percentage differences between the timepoints are presented in table 8.

Table 5: *Sodium concentrations after delayed freezing of plasma: Descriptive values.*

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0 h	139.5	2.0	136	138	140	141	145	9
4 h	139.6	2.0	136	138	139	141	144	8
24 h	140.1	1.8	137	139	140	141	144	7
48 h	139.7	2.1	136	138	140	141	144	8
72 h	140.3	1.9	136	139	141	141	144	8





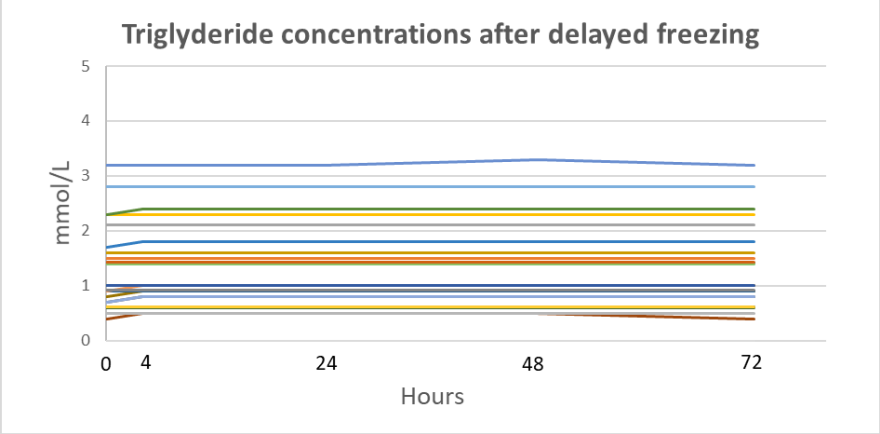


Figure 6: Measured sodium, cholesterol and triglyceride concentrations in EDTA-plasma for the 24 participants after 0, 4, 24, 48 and 72 h of delayed freezing.

Table 6: Cholesterol concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0 h	5.53	0.85	3.6	5.13	5.50	6.05	7.3	3.7
4 h	5.56	0.85	3.6	5.13	5.45	6.15	7.3	3.7
24 h	5.58	0.87	3.6	5.15	5.50	6.08	7.3	3.7
48 h	5.56	0.85	3.7	5.13	5.50	6.13	7.4	3.7
72 h	5.55	0.80	3.8	5.23	5.45	6.15	7.2	3.4

Table 7: Triglyceride concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0 h	1.30	0.76	0.4	0.7	1.0	1.68	3.2	2.8
4 h	1.33	0.76	0.5	0.8	1.0	1.75	3.2	2.7
24 h	1.33	0.76	0.5	0.8	1.0	1.75	3.2	2.7
48 h	1.34	0.77	0.5	0.8	1.0	1.75	3.3	2.8
72 h	1.33	0.76	0.4	0.8	1.0	1.75	3.2	2.8

Table 8: Percentage differences in mean concentrations for sodium, cholesterol and triglycerides after delayed freezing of EDTA-plasma.

Analyte	4 vs 0 h	24 vs 0 h	48 vs 0 h	72 vs 0 h	24 vs 4 h	48 vs 4 h	72 vs 4 h	48 vs 24 h	72 vs 24 h	72 vs 48 h
Sodium	0.1	0.4	0.1	0.6	0.4	0.1	0.5	-0.3	0.1	0.4
Cholesterol	0.5	0.9	0.5	0.4	0.4	0.0	0.2	-0.4	-0.5	-0.2
Triglycerides	2.3	2.3	3.1	2.3	0.0	0.8	0.0	0.8	0.0	-0.8

Cholesterol concentrations were normally distributed while sodium and triglycerides weren't and for them the analysis to compare the effects of delayed freezing of EDTA-plasma on sodium and triglyceride concentrations at different timepoints was performed using the non-parametric Friedman test.

The percentage differences between the timepoints for freezing of the plasma is small with less than 1 % for sodium and cholesterol and a maximum of 3.1 % for triglycerides. Nevertheless, the statistical analysis revealed significant group differences between at least two timepoints for sodium and triglycerides, $\chi2(4) = 13.433$, $p=0.009$ and $\chi2(4) = 24.235$ p=<0,001, respectively.

For triglycerides, there tends to be an increase in concentrations between 0 and 4 h of delayed freezing before it is stabilized on a higher level. Post-hoc analysis with Wilcoxon signed-rank test was performed to compare two and two timepoints with a Bonferroni correction to adjust for multiple testing. There was a statistically significant difference between triglyceride concentrations at 0 h and 48 h delayed freezing of EDTA-plasma, z= -2.828, p=0.047, but not for any other groups.

The mean sodium concentrations have a tendency to increase with increased delay. However, the post-hoc analysis revealed no statistically significant differences between the timepoints with the biggest differences, 4 vs. 72 hours and 0 vs. 72 hours, p=0.055 and p=0.105, respectively, after correction for multiple comparisons.

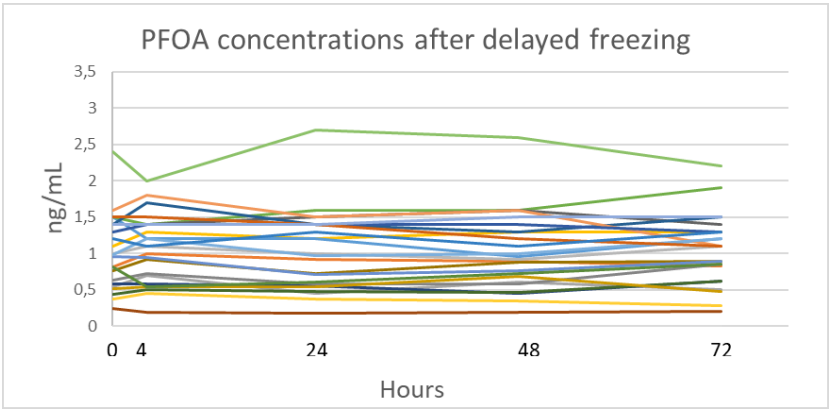
When it comes to cholesterol, there was not a statistically significant difference between the concentrations at the different timepoints for delayed freezing according to the Oneway repeated measures ANOVA, F (4,23) =1.487, p=0.213.

3.1.2 PFAS

All samples were analyzed for 23 different PFAS; PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDa, PFTrDa, PFTeDa, PFBS, PFHxS, PFHpS, PFOS, PFDS, PFOSA, MeFOSA, EtFOSA, HFPO-DA (GenX), ADONA, 6:2 Cl-PFESA and 8:2 Cl-PFESA. However, only 11 of them were detected above the LOQ in one or more samples. Results for these 11 are presented below.

PFOA, PFNA, PFHxS and PFOS were detected in all the 120 samples. The measured concentrations are shown in figure 7 a, b, c and d, respectively, while the descriptive values are presented in table 9 for PFOA, table 10 for PFNA, table 11 for PFHxS and table 12 for PFOS.

a)



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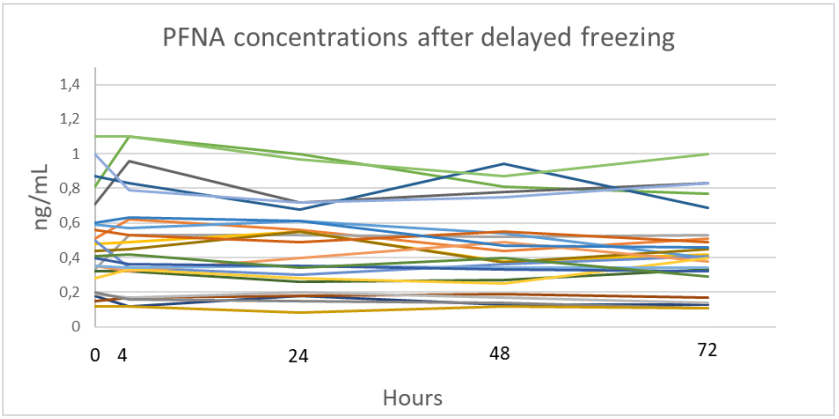
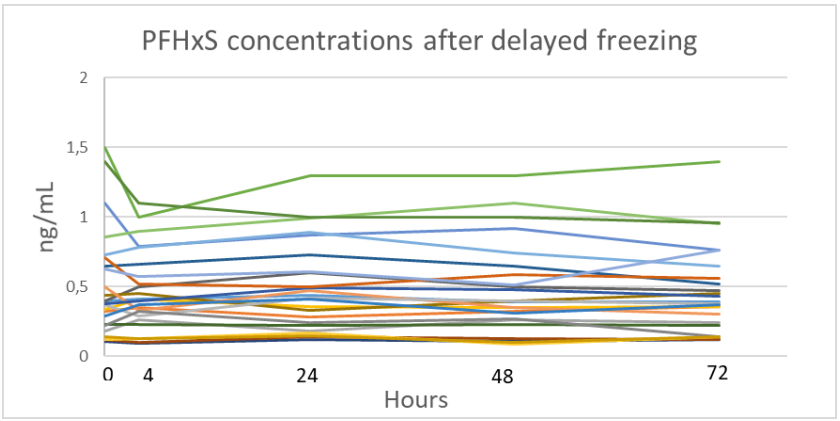


c)



d)

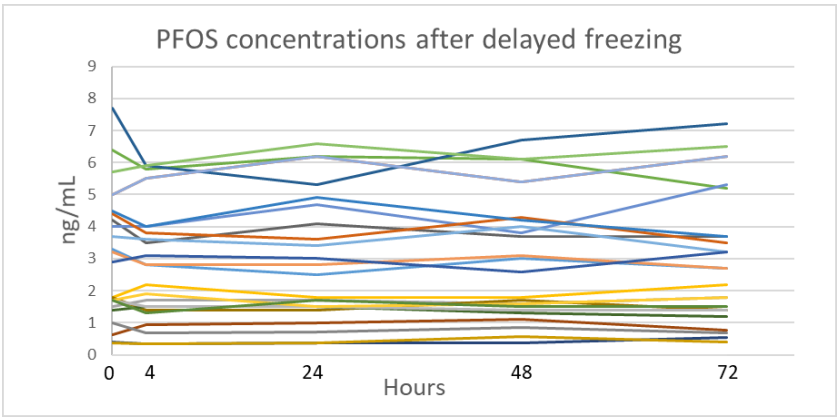


Figure 7: Measured PFOA, PFNA, PFHxS and PFOS concentrations for the 24 participants after delayed freezing of EDTA-plasma.

Table 9: PFOA concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	1.025	0.492	0.25	0.595	0.990	1.40	2.4	2.15
4 h	1.073	0.469	0.20	0.617	1.100	1.40	2.0	1.80
24 h	1.030	0.554	0.18	0.565	0.985	1.40	2.7	2.52
48 h	1.044	0.534	0.20	0.630	0.960	1.38	2.6	2.40
72 h	1.052	0.481	0.21	0.672	1.100	1.30	2.2	1.99

Table 10: PFNA concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	0.477	0.266	0.12	0.290	0.425	0.597	1.1	0.98
4 h	0.490	0.292	0.12	0.320	0.435	0.628	1.1	0.98
24 h	0.461	0.248	0.08	0.265	0.445	0.610	1.0	0.92
48 h	0.442	0.242	0.12	0.255	0.390	0.548	0.91	0.79
72 h	0.438	0.242	0.11	0.297	0.405	0.525	1.0	0.89

Table 11: PFHxS concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	0.506	0.385	0.11	0.223	0.390	0.695	1.5	1.39
4 h	0.462	0.282	0.094	0.267	0.405	0.637	1.1	1.01
24 h	0.496	0.321	0.12	0.225	0.430	0.700	1.3	1.18
48 h	0.479	0.327	0.084	0.263	0.395	0.635	1.3	1.22
72 h	0.466	0.317	0.12	0.225	0.385	0.628	1.4	1.28

Table 12: PFOS concentrations after delayed freezing of plasma: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	3.08	2.00	0.38	1.55	3.05	4.48	7.7	7.32
4 h	2.92	1.84	0.33	1.43	2.80	4.00	5.9	5.57
24 h	3.04	2.03	0.36	1.50	2.65	4.85	6.6	6.24
48 h	3.01	1.93	0.38	1.43	2.80	4.28	6.7	6.32
72 h	3.04	2.08	0.55	1.40	2.70	4.83	7.2	6.65

Seven of the PFAS were measured above LOQ in some of the 120 samples; PFDA (measured in 105 samples), PFUnDA (88), PFHpA (56), PFDoDA (21), PFTrDA (29), PFHpS (64) and 6:2 Cl-PFESA (42) and the results are shown in figure 8 a-g.

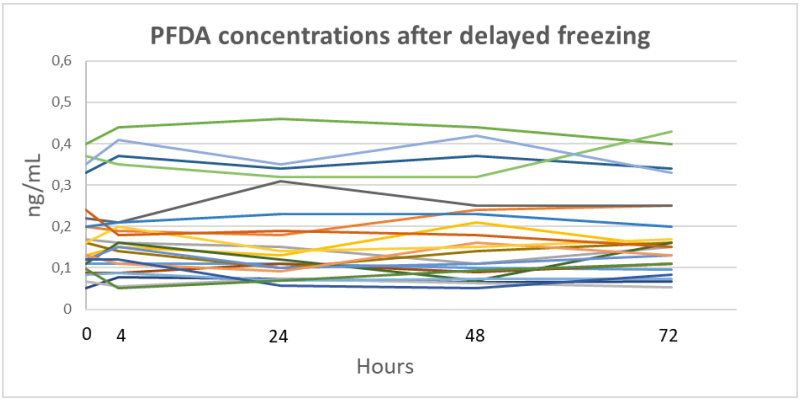
For the statistical analysis, if a participant had samples without reported concentration above LOQ at one or more timepoints, the LOQ was used for these samples. Participants without any measurements above LOQ for an analyte were excluded from further analysis of that particular analyte. The descriptive values and the number of samples reported with concentrations above LOQ for each of the timepoints are presented in table 13 for PFDA, table 14 for PFUnDA, table 15 for PFHpA, table 16 for PFDODA, table 17 for PFTrDA, table 18 for PFHpS and table 19 for 6:2 Cl-PFESA. The percentage differences between the timepoints for all PFAS are presented in table 20.

Table 13: PFDA concentrations after delayed freezing of plasma: Descriptive values.

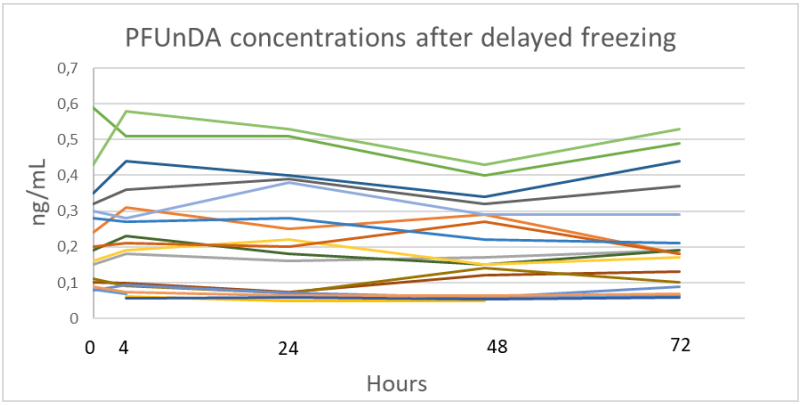
Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	21	0.178	0.102	0.050[a]	0.107	0.145	0.225	0.40	0.350[b]
4 h	21	0.183	0.113	0.050[a]	0.104	0.155	0.210	0.44	0.390[b]
24 h	22	0.172	0.114	0.058	0.087	0.125	0.250	0.46	0.402
48 h	21	0.179	0.118	0.050[a]	0.086	0.145	0.243	0.44	0.390[b]
72 h	22	0.182	0.108	0.053	0.107	0.150	0.250	0.43	0.377

[a]: LOQ is used for one sample which was not detected above LOQ
[b]: LOQ is used as minimum for one sample which was not detected above LOQ

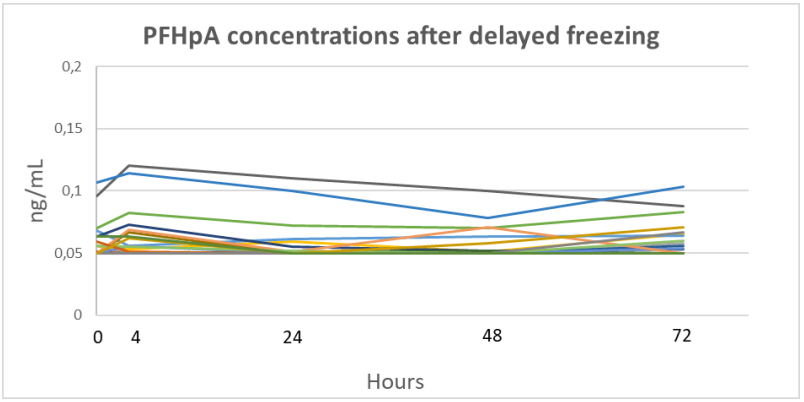
a)


PFDA concentrations after delayed freezing
ng/mL
0,6
0,5
0,4
0,3
0,2
0,1
0
0
4
24
48
72
Hours

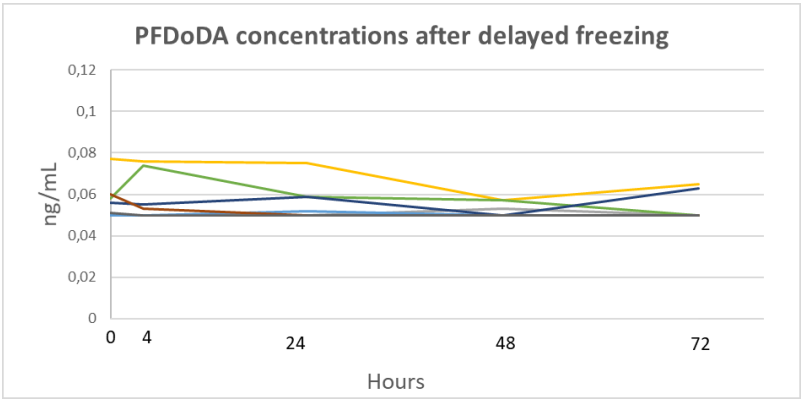
b)


PFUnDA concentrations after delayed freezing
ng/mL
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
4
24
48
72
Hours

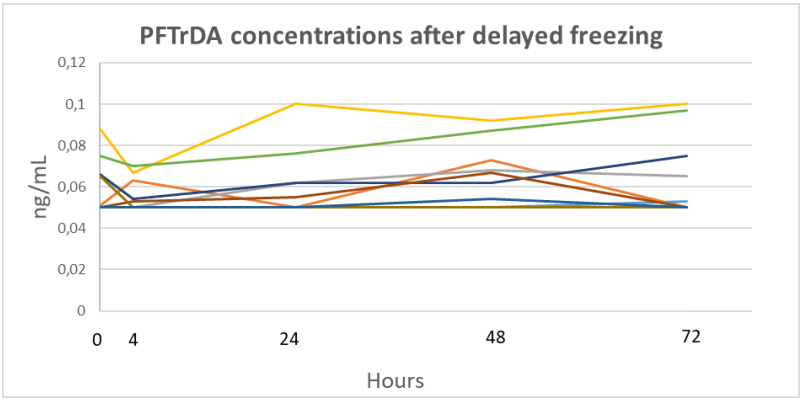
c)


PFHpA concentrations after delayed freezing
ng/mL
0,2
0,15
0,1
0,05
0
0
4
24
48
72
Hours

d)


PFDoDA concentrations after delayed freezing
ng/mL
0,12
0,1
0,08
0,06
0,04
0,02
0
0
4
24
48
72
Hours

e)



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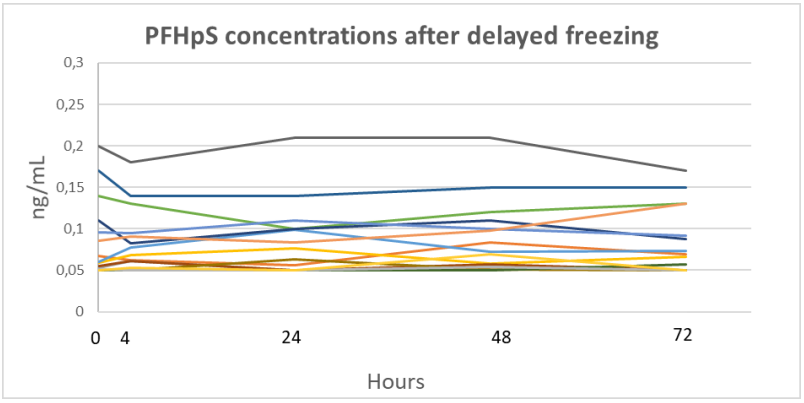

g)

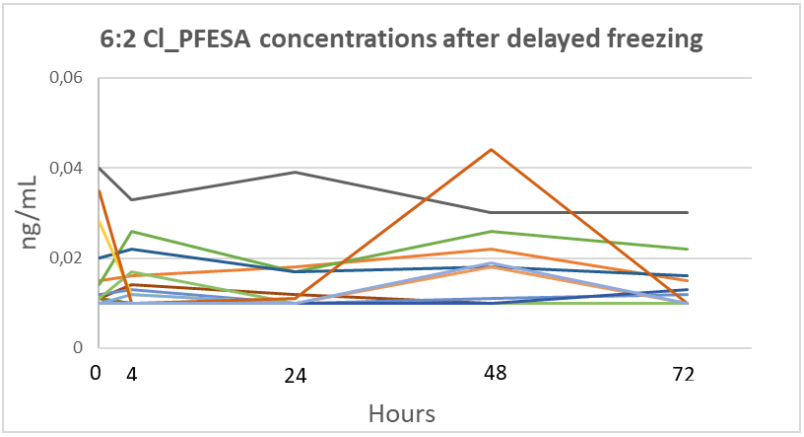

Figure 8: Measured PFDA, PFUnDA, PFHpA, PFDoDA PFTrDA, PFHpS and 6:2 Cl-PFESA concentrations for the 24 participants after delayed freezing of EDTA-plasma after 0, 4, 24, 48 and 72 h.

Table 14: PFUnDA concentrations after delayed freezing of plasma: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	16	0.201	0.147	0.050[a]	0.081	0.160	0.300	0.59	0.540[b]
4 h	19	0.219	0.161	0.057	0.073	0.190	0.310	0.58	0.523
24 h	17	0.210	0.163	0.050[a]	0.065	0.180	0.380	0.53	0.480[b]
48 h	18	0.191	0.126	0.050[a]	0.061	0.150	0.290	0.43	0.380[b]
72 h	18	0.203	0.152	0.050[a]	0.070	0.180	0.290	0.53	0.480[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 15: PFHpA concentrations after delayed freezing of plasma: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	11	0.0608	0.0163	.050[a]	0.050	0.056	0.064	0.107	0.053[b]
4 h	14	0.0651	0.0211	0.050[a]	0.050	0.056	0.070	0.120	0.070[b]
24 h	10	0.0589	0.0178	0.050[a]	0.050	0.051	0.060	0.110	0.060[b]
48 h	9	0.0581	0.0178	0.050[a]	0.050	0.051	0.065	0.100	0.050[b]
72 h	12	0.0623	0.0137	0.050[a]	0.050	0.057	0.068	0.103	0.053[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 16: PFDoDA concentrations after delayed freezing of plasma: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	8	0.0565	0.0092	0.050[a]	0.050	0.0535	0.060	0.077	0.027[b]
4 h	4	0.0573	0.0111	0.050[a]	0.050	0.0515	0.069	0.076	0.026[b]
24 h	4	0.0556	0.0088	0.050[a]	0.050	0.0510	0.059	0.075	0.025[b]
48 h	3	0.0521	0.0032	0.050[a]	0.050	0.0500	0.056	0.057	0.007[b]
72 h	2	0.0535	0.0065	0.050[a]	0.050	0.0500	0.060	0.063	0.013[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 17: PFTrDA concentration after delayed freezing of plasma: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	6	0.0595	0.0135	0.050[a]	0.050	0.051	0.0682	0.088	0.038[b]
4 h	5	0.0557	0.0079	0.050[a]	0.050	0.052	0.0640	0.070	0.020[b]
24 h	5	0.0605	0.0163	0.050[a]	0.050	0.053	0.0655	0.100	0.050[b]
48 h	8	0.0633	0.0153	0.050[a]	0.050	0.065	0.0765	0.092	0.042[b]
72 h	5	0.0640	0.0200	0.050[a]	0.050	0.052	0.0805	0.100	0.050[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 18: PFHpS concentration after delayed freezing of plasma: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	13	0.0886	0.0473	0.050[a]	0.0515	0.0635	0.117	0.20	0.15[b]
4 h	13	0.0815	0.0381	0.050[a]	0.0530	0.0650	0.094	0.18	0.13[b]
24 h	11	0.0874	0.0432	0.050[a]	0.0500	0.0800	0.108	0.21	0.16[b]
48 h	15	0.0898	0.0434	0.050[a]	0.0547	0.0780	0.108	0.21	0.16[b]
72 h	12	0.0859	0.0395	0.050[a]	0.0500	0.0710	0.123	0.17	0.12[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 19: 6:2 Cl-PFESA concentration after delayed freezing of plasma: Descriptive values.

	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0 h	11	0.0165	0.0099	0.010[a]	0.010	0.011	0.020	0.040	0.030[b]
4 h	9	0.0150	0.0069	0.010[a]	0.010	0.012	0.017	0.033	0.023[b]
24 h	6	0.0136	0.0076	0.010[a]	0.010	0.010	0.017	0.039	0.029[b]
48 h	9	0.0172	0.0100	0.010[a]	0.010	0.011	0.022	0.044	0.034[b]
72 h	7	0.0132	0.0058	0.010[a]	0.010	0.010	0.015	0.030	0.020[b]

[a]: LOQ is used for one or more samples when concentration was not detected above LOQ
[b]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 20: Percentage differences in mean concentrations for PFAS after delayed freezing of EDTA-plasma.

Analyte	4 vs 0 h	24 vs 0 h	48 vs 0 h	72 vs 0 h	24 vs 4 h	48 vs 4 h	72 vs 4 h	48 vs 24 h	72 vs 24 h	72 vs 48 h
PFOA	4.7	0.5	1.9	2.6	-4.0	-2.7	-2.0	1.4	2.1	0.8
PFNA	2.8	-3.4	-7.3	-8.2	-5.9	-9.8	-10.6	-4.1	-5.0	-0.9
PFHxS	-8,7	-2,0	-5,3	-7,9	7,4	3,7	0,9	-3,4	-6,0	-2,7
PFOS	-5,2	-1,3	-2,3	-1,3	4,1	3,1	4,1	-1,0	0,0	1,0
PFDA	2,8	-3,4	0,6	2,2	-6,0	-2,2	-0,5	4,1	5,8	1,7
PFUnDA	9,0	4,5	-5,0	1,0	-4,1	-12,8	-7,3	-9,0	-3,3	6,3
PFHpA	7,1	-3,1	-4,4	2,5	-9,5	-10,8	-4,3	-1,4	5,8	7,2
PFDoDA	1,4	-1,6	-7,8	-5,3	-3,0	-9,1	-6,6	-6,3	-3,8	2,7
PFTrDA	-6,4	1,7	6,4	7,6	8,6	13,6	14,9	4,6	5,8	1,1
PFHpS	-8,0	-1,4	1,4	-3,0	7,2	10,2	5,4	2,7	-1,7	-4,3
6:2 Cl-PFESA	-9,1	-17,6	4,2	-20,0	-9,3	14,7	-12,0	26,5	-2,9	-23,3

PFNA, PFDA, PFHpA, PFDoDA and PFTrDA were normally distributed and the Oneway repeated measures ANOVA was used to compare the effects of delayed freezing og EDTA-plasma on the analyte level, while the rest of the detected PFAS were not and the non-parametric Friedman test was used.

The analysis revealed that there were only statistically significant differences between timepoints for PFNA and PFTrDA, F (4,23) = 2.510, p=0.047 and F (4,9) =2.650, p=0.049, respectively.

The mean and median PFNA concentrations tend to decrease with delayed freezing of EDTA-plasma. However, there was not a statistically significant difference between PFNA concentrations when comparing two and two timepoints, not even for the timepoints with the largest difference in means, 4 and 72 hours, t=2.622, p=0.102.

For PFTrDA, there were only 29 samples belonging to 10 of the participants with concentrations above LOQ, but the mean concentrations had a tendency to increase with longer delays. Analyzing the difference between two and two timepoints for PFTrDA, for the timepoints with the highest difference, 4 and 48 h, there was not a statistically significant difference in PFtrDA concentrations, t=2.900, p=0.063.

PFDoDA was one of the analytes which did not have a statistically significant difference between concentrations at 0, 4, 24, 48 and 72 h of delayed freezing, F (4,8) =1.577, p=0.208. However, the concentrations above LOQ of 0.05 ng/mL was only measured in 21 of the 120 samples belonging to 9 participants. Nevertheless, there were decreasing numbers of samples above the LOQ with increasing delay; 8 of the samples at 0 h, and in 4, 4, 3 and 2 of the samples at 4, 24, 48 and 72 h, respectively.

3.2 Delayed separation of blood

The measured concentrations for each sample at each timepoint are found in Appendix 2. For sodium, cholesterol and triglycerides, four samples went through one additional freeze-thaw cycle before analysis and were analyzed at different timepoints than the other samples from the participant: id 13, 24h, id 16, 24h and id 24, 48 and 72h.

When analyzing the reported results in this part of the project, two possible sample switches were detected. For all samples, it is suspected that ID 12 at 72 h and ID 16 at 48 h have been switched. Even if the time from sample collection until centrifugation were differently for the two samples, they were processed at the same day at about the same

time before freezing them at -80 °C. This switch has affected the interpretation of the results while they contribute to the values at different timepoints.

In addition, it is suspected that the 4 h samples from id 17-20 were switched for the aliquot that was shipped to Department of Medical Biochemistry at Oslo University Hospital. Based on the reported concentrations, it is suspected that either sample-pairs 17 and 20, and 18 and 19 are switched or that there was a shift causing sample 17 to be marked as sample 20, sample 18 to be marked as sample 17, sample 19 to be marked as sample 18 and sample 20 to be marked as sample 19. For cholesterol and triglycerides, the results are the same regardless of which of the hypothesis is correct. For sodium, it would make a small difference for the results for each participant, but not for the statistical analysis when comparing the different timepoints. However, the latter hypothesis is used based on the probability of the deviation based on the practical sample handling in the laboratory.

3.2.1 Sodium, cholesterol and triglycerides

Figure 9, 10 and 11 displays the reported and corrected sodium, cholesterol and triglyceride concentrations in EDTA-plasma after delayed centrifugation of blood and table 21, 22 and 23 presents the descriptive values for the reported and corrected concentrations. The percentage differences between the timepoints are presented in table 24 and for 48 and 72 h, based on the adjusted concentrations.

Table 21: Reported and adjusted sodium concentrations: Descriptive values.

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0.5 h	138.0	1.3	135	137	138	139	140	5
4 h	139.1	1.5	136	138	139	140	142	6
24 h	140.2	3.3	136	138	139.5	141	152	16
48 h[a]	139.3	1.9	135	138	139	141	142	7
48 h[b]	139.4	2.0	135	138	139.5	141	142	7
72 h[a]	139.2	1.9	135	138	139.5	141	142	7
72 h[b]	139.1	1.8	135	139	139	140	142	7

[a]: Original concentrations
[b]: Adjusted concentrations

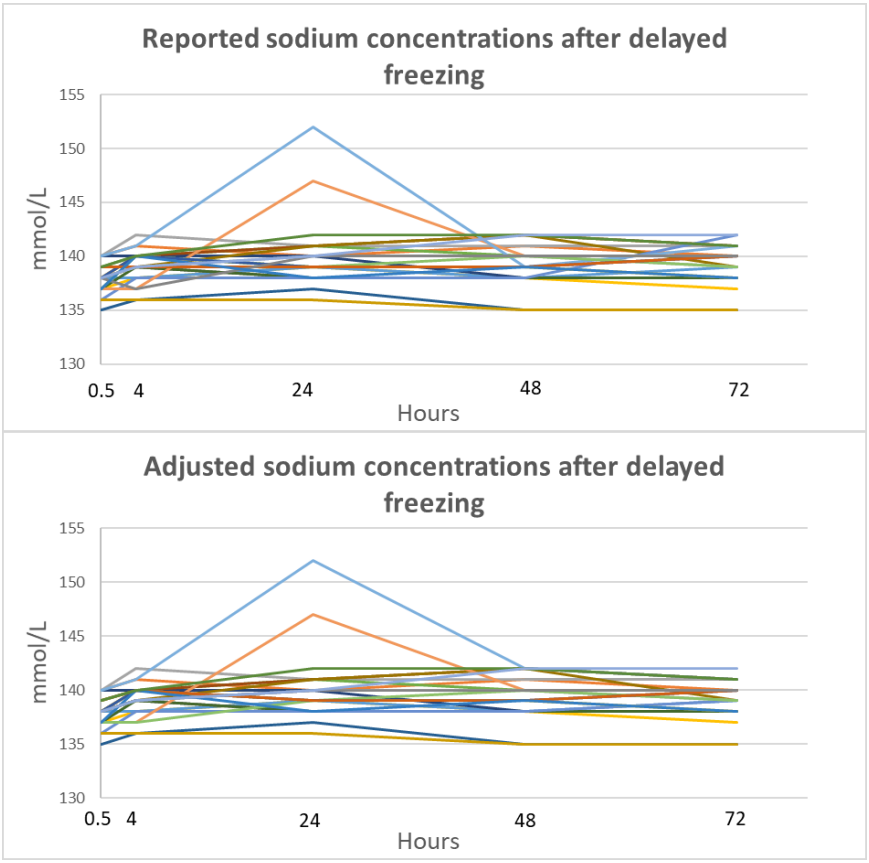

Figure 9: Reported and adjusted sodium concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 22: Reported and adjusted cholesterol concentrations after delayed centrifugation: Descriptive values.

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0.5 h	5.12	1.11	3.2	4.23	5.15	5.98	7.3	4.1
4 h	5.16	1.13	3.1	4.33	5.20	6.13	7.3	4.2
24 h	5.27	1.16	3.2	4.40	5.20	6.18	7.4	4.2
48 h[a]	5.29	1.69	3.2	4.50	5.25	6.18	7.4	4.2
48 h[b]	5.27	1.14	3.2	4.50	5.25	6.18	7.4	4.2
72 h[a]	5.27	1.10	3.2	4.45	5.25	6.20	7.3	4.1
72 h[b]	5.29	1.13	3.2	4.45	5.25	6.20	7.3	4.1

[a]: Original concentrations
[b]: Adjusted concentrations

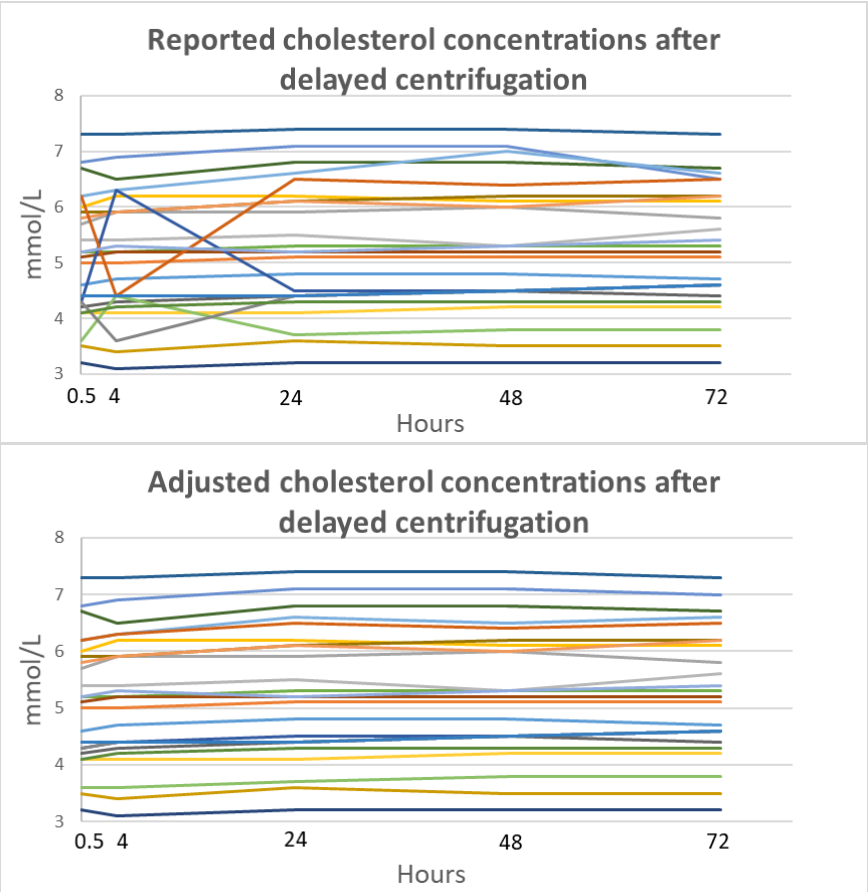

Figure 10: Reported and adjusted cholesterol concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 23: Reported and adjusted triglyceride concentrations after delayed centrifugation: Descriptive values.

Delay	Mean mmol/L	Std. dev mmol/L	Min. mmol/L	Q1 mmol/L	Median mmol/L	Q3 mmol/L	Max. mmol/L	Range mmol/L
0.5 h	1.18	0.62	0.4	0.80	0.95	1.50	2.8	2.4
4 h	1.19	0.58	0.5	0.80	1.00	1.48	2.5	2.0
24 h	1.21	0.61	0.5	0.80	0.95	1.50	2.8	2.3
48 h[a]	1.23	0.66	0.5	0.80	0.95	1.58	2.7	2.2
48 h[b]	1.19	0.61	0.5	0.80	0.95	1.50	2.7	2.2
72 h[a]	1.19	0.57	0.5	0.80	0.95	1.50	2.8	2.3
72 h[b]	1.20	0.62	0.5	0.80	0.95	1.50	2.8	2.3

[a]: Original concentrations
[b]: Adjusted concentrations

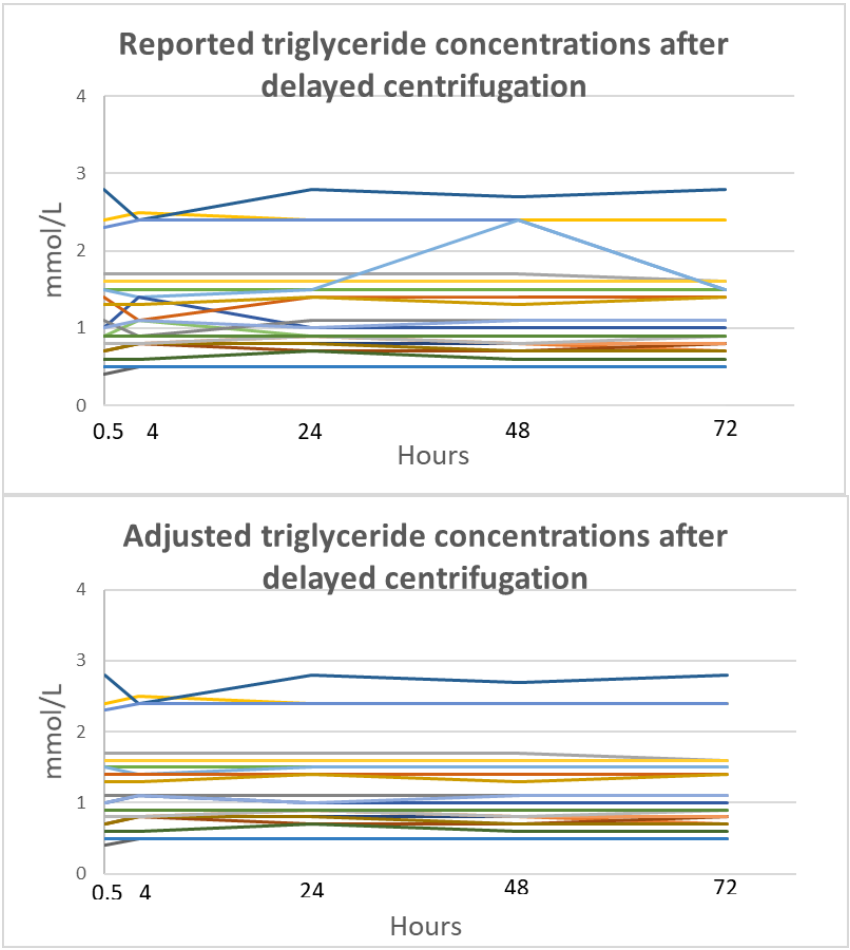

Figure 11: Reported and adjusted triglyceride concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 24: Percentage differences in sodium, cholesterol and triglyceride mean concentrations after delayed centrifugation of blood.

Analyte	4 vs 0.5 h	24 vs 0.5 h	48 vs 0.5 h	72 vs 0.5 h	24 vs 4 h	48 vs 4 h	72 vs 4 h	48 vs 24 h	72 vs 24 h	72 vs 48 h
Sodium	0,8	1,6	1,0	0,8	0,8	0,2	0,0	-0,6	-0,8	-0,2
Cholesterol	0,8	2,9	2,9	3,3	2,1	2,1	2,5	0,0	0,4	0,4
Triglycerides	0,8	2,5	0,8	1,7	1,7	0,0	0,8	-1,7	-0,8	0,8

[a] The adjusted concentrations were used.

Investigation of the effect of delayed separation of blood after 0.5, 4, 24, 48 and 72 h on analyte concentrations in EDTA plasma revealed that there were differences between timepoints for sodium and cholesterol, and it did not matter whether the reported or adjusted concentrations were used. For triglycerides, there was not a statistically significant difference between timepoints neither using the reported nor adjusted concentrations according to the non-parametric Friedman test, $\chi2(4) = 5.399$, $p=0.249$ and $\chi2(4) = 7.757$, $p=0.101$, respectively.

For sodium, the mean and median concentrations were lower for samples which were centrifuged after 30 min, compared to the other timepoints. Except for the mean for samples centrifuged after 24 h where two samples may be defined as outliers, the mean concentrations seem to have been stabilized on a new, approximately 1 % higher level. Post-hoc Wilcoxon signed test with Bonferroni correction to adjust for multiple testing was used for comparison of two and two timepoints and the increased concentrations from 0.5 h and to each of the other timepoints were statistically significant.

Regarding cholesterol, the mean and median concentrations increased slightly with prolonged delay of centrifugation and were 3.3% higher at 72 h storage before centrifugation compared to 0.5 h. To evaluate the effect of delayed centrifugation on the adjusted cholesterol levels, One Way repeated measures ANOVA was conducted, and there was a statistically difference between timepoints ($F(4,23) = 26.096$, $p<.001$. There are statistically significant differences between cholesterol concentrations between centrifugation after 30 min and 24, 48 and 72 h corresponding to an increase of 2.9, 2.9 and 3.3 %, respectively, and after 4 h and 24, 48 and 72 h, corresponding to an increase of 2.1, 2.1 and 2.5 %, respectively, all $p<.001$.

When the reported concentrations were used, the analysis revealed similar results. There was a statistically significant difference at group level, $\chi2(4) = 40,585$, $p<.001$ according to the non-parametric Friedman test. Further investigation revealed that there were statistically significant differences in cholesterol levels between centrifugation after 0.5 h and centrifugation after 24, 48 and 72 h, p>,001 $p<.001$ and $p=0.008$, respectively, corresponding to increases of 2.9, 3.3 and 2.9 %.

3.2.2 PFAS

All samples were analyzed for 23 different PFAS; PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDa, PFTrDa, PFTeDa, PFBS, PFHxS, PFHpS, PFOS, PFDS, PFOSA, MeFOSA, EtFOSA, HFPO-DA (GenX), ADONA, 6:2 Cl-PFESA and 8:2 Cl-PFESA but only 11 of them, the same as in the delayed freezing part of this project, were detected above the LOQ in one or more samples. PFDoDA and PFTrDA were only

measured in one and three of the samples, respectively, and no further analysis of the data has been done. Results for the remaining 9 are presented below.

PFOA, PFNA, PFHxS and PFOS were detected in all samples, and the concentrations in the five samples from each participant are presented in figure 12 for PFOA, figure 13 for PFNA, figure 14 for PFHxS and figure 15 for PFOS and the descriptive statistics in table 25 for PFOA, table 26 for PFNA, table 27 for PFHxS and table 28 for PFOS.

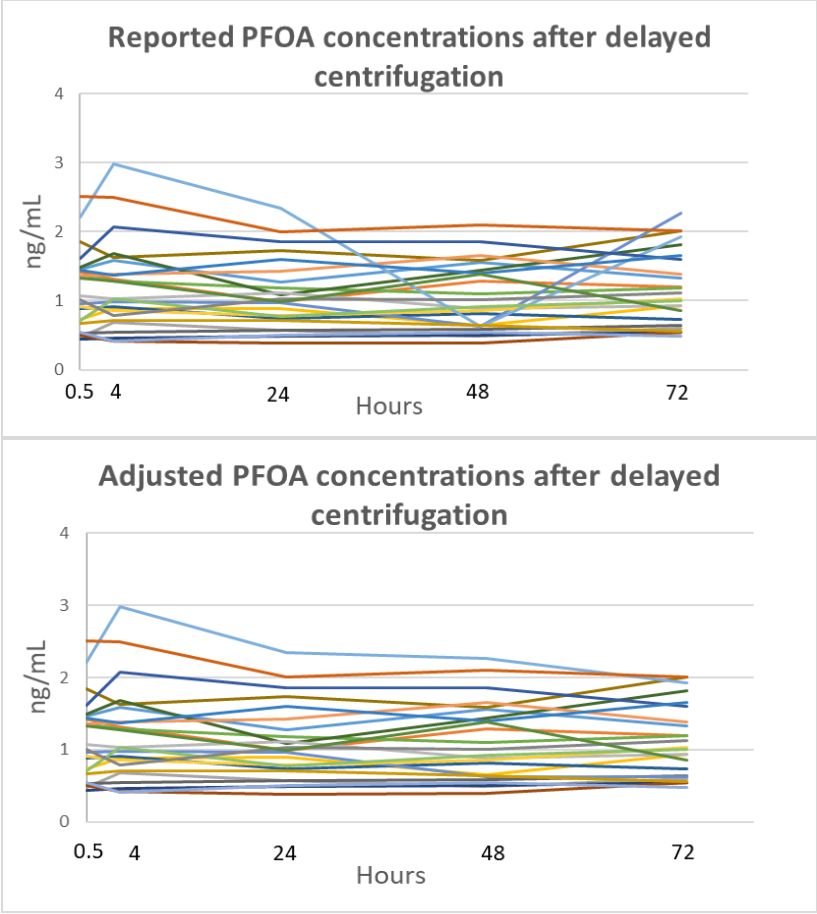

Figure 12: Reported and adjusted PFOA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 25: Reported and adjusted PFOA concentrations after delayed centrifugation: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	1.145	0.555	0.44	0.68	1.04	1.46	2.51	2.07
4 h	1.200	0.645	0.41	0.73	1.06	1.53	2.98	2.57
24 h	1.083	0.515	0.38	0.72	0.99	1.39	2.34	1.96
48 h [a]	1.038	0.488	0.39	0.63	0.90	1.43	2.10	1.71
48 h [b]	1.063	0.540	0.39	0.63	0.97	1.53	2.27	1.88
72 h [a]	1.182	0.543	0.48	0.66	1.08	1.65	2.27	1.79
72 h [b]	1.113	0.502	0.48	0.63	1.02	1.54	2.01	1.53

[a]: Original concentrations
[b]: Adjusted concentrations

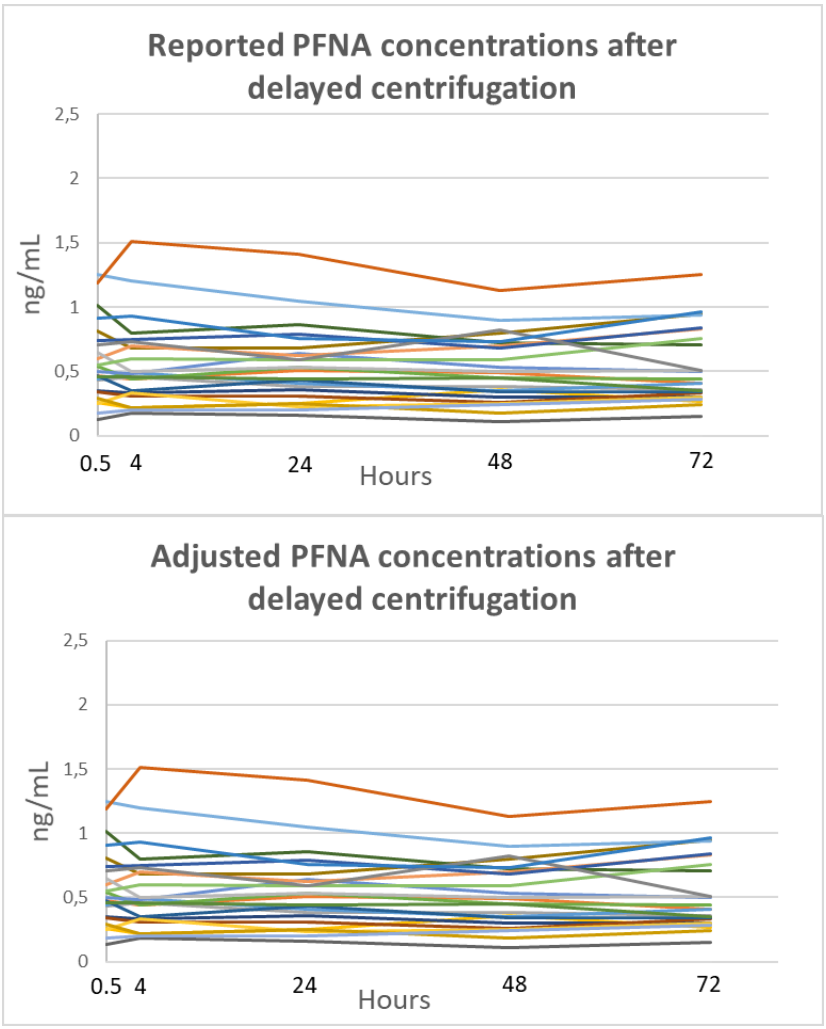

Figure 13: Reported and adjusted PFNA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 26: Reported and adjusted PFNA concentrations after delayed centrifugation: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	0.564	0.300	0.13	0.34	0.49	0.73	1.25	1.12
4 h	0.555	0.322	0.18	0.33	0.47	0.72	1.51	1.33
24 h	0.541	0.291	0.16	0.32	0.52	0.67	1.41	1.25
48 h [a]	0.494	0.241	0.11	0.31	0.47	0.70	1.13	1.02
48 h [b]	0.511	0.255	0.11	0.31	0.47	0.72	1.13	1.02
72 h [a]	0.556	0.301	0.15	0.30	0.43	0.84	1.25	1.10
72 h [b]	0.539	0.292	0.15	0.31	0.43	0.82	1.25	1.10

[a]: Original concentrations
[b]: Adjusted concentrations

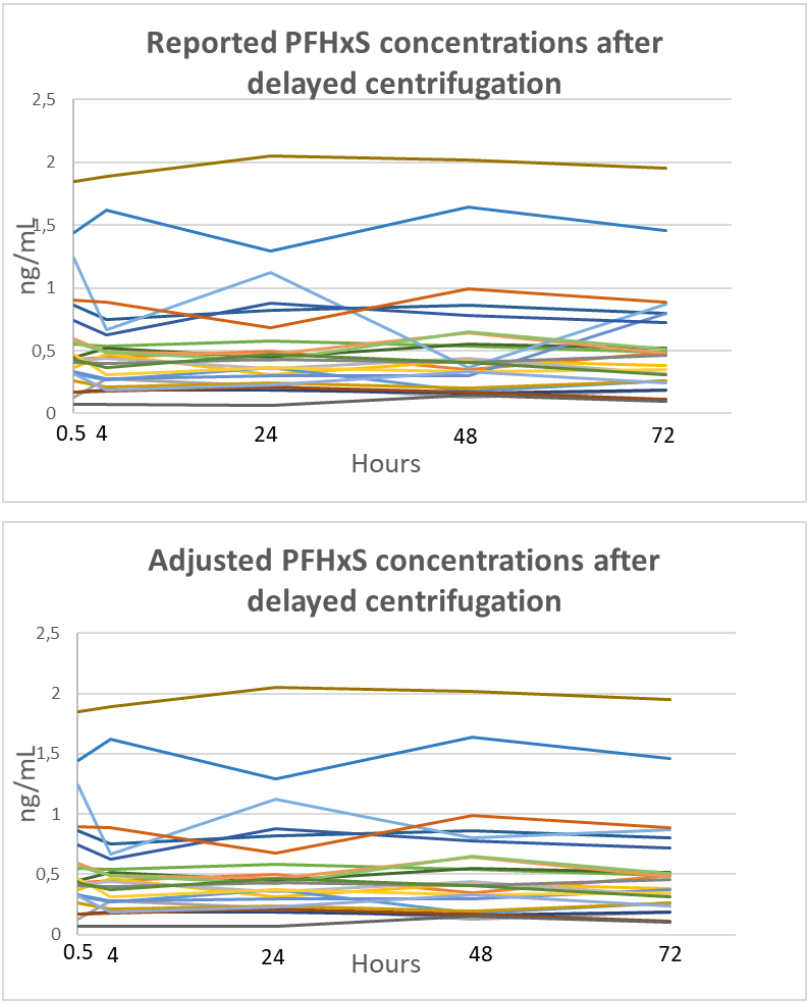

Figure 14: Reported and adjusted PFHxS concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 27: Reported and adjusted PFHxS concentrations after delayed centrifugation: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	0.562	0.430	0.07	0.32	0.43	0.70	1.85	1.78
4 h	0.525	0.428	0.07	0.27	0.45	0.61	1.89	1.82
24 h	0.543	0.437	0.07	0.26	0.43	0.66	2.05	1.98
48 h [a]	0.542	0.462	0.13	0.23	0.41	0.65	2.05	1.92
48 h [b]	0.560	0.463	0.13	0.23	0.42	0.75	2.05	1.92
72 h [a]	0.548	0.431	0.10	0.26	0.47	0.78	1.95	1.85
72 h [b]	0.530	0.429	0.10	0.26	0.42	0.67	1.95	1.85

[a]: Original concentrations
[b]: Adjusted concentrations

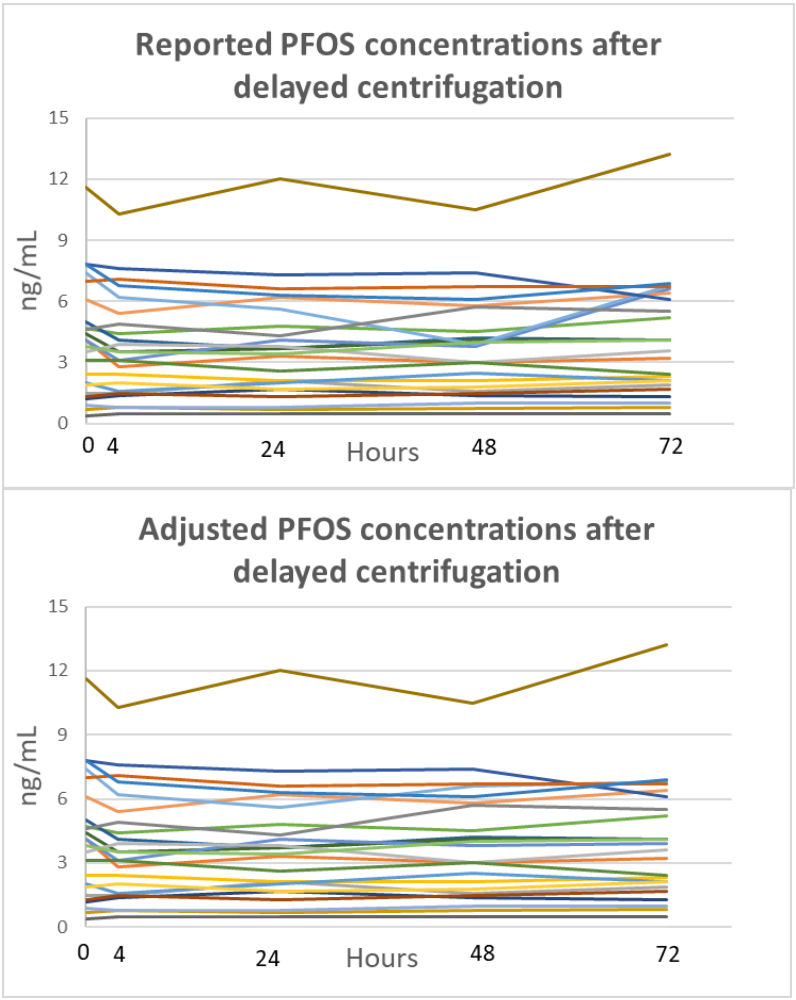

Figure 15: Reported and adjusted PFOS concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 28: Reported and adjusted PFOS concentrations after delayed centrifugation: Descriptive values.

Delay	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	4.05	2.80	0.4	1.6	3.9	5.8	11.6	11.2
4 h	3.72	2.50	0.5	1.5	3.3	5.3	10.3	9.8
24 h	3.77	2.64	0.5	1.8	3.6	5.4	12.0	11.5
48 h [a]	3.70	2.42	0.5	1.7	3.4	5.4	10.5	10.0
48 h [b]	3.81	2.49	0.5	1.7	3.4	5.8	10.5	10.0
72 h [a]	4.11	2.88	0.5	2.0	3.9	6.3	13.2	12.7
72 h [b]	4.00	2.83	0.5	2.0	3.8	6.0	13.2	12.7

[a]: Original concentrations
[b]: Adjusted concentrations

PFDA and PFUnDA were detected above LOQ in most of the samples, 114 and 113, respectively, and PFHpA, PFHpS and 6:2 Cl-PFESA were reported in 41, 72 and 73 samples. Visualization of the concentrations are presented in figure 16 for PFDA, figure 17 for PFUnDA, figure 18 for PFHpA, figure 19 for PFHpS and figure 20 for 6:2 Cl-PFESA.

For the statistical analysis, if a participant had samples without reported concentration above LOQ at one or more timepoints, the LOQ was used for these samples. Participants without any measurements above LOQ for an analyte were excluded from further analysis of that particular analyte. The descriptive values for reported and adjusted data and the number of samples with concentrations above LOQ for each of the timepoints for delayed separation are presented in table 29 for PFDA, table 30 for PFUnDA, table 31 for PFHpA, table 32 for PFHpS and table 33 for 6:2 Cl-PFESA. In table 34 the percentage differences between the various timepoints of delayed centrifugation are presented. The adjusted concentrations were used.

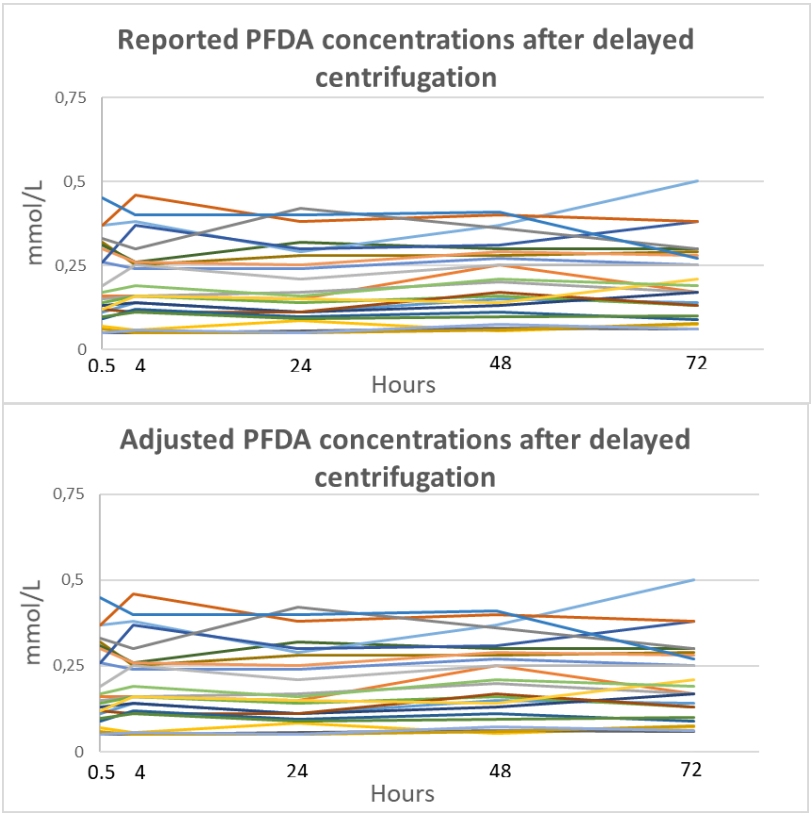

Figure 16: Reported and adjusted PFDA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 29: Reported and adjusted PFDA concentrations after delayed centrifugation: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	22	0.195	0.118	0.050[c]	0.101	0.155	0.308	0.45	0.40[d]
4 h	22	0.201	0.117	0.050[c]	0.113	0.160	0.260	0.46	0.41[d]
24 h	22	0.192	0.114	0.050[c]	0.100	0.155	0.287	0.42	0.37[d]
48 h [a]	24	0.208	0.108	0.054	0.115	0.205	0.287	0.41	0.36
48 h [b]	24	0.213	0.112	0.054	0.115	0.205	0.297	0.41	0.36
72 h [a]	24	0.212	0.120	0.059	0.108	0.180	0.287	0.50	0.45
72 h [b]	24	0.207	0.116	0.059	0.108	0.180	0.287	0.50	0.45

a: Original concentrations
b: Adjusted concentrations
[c]: LOQ is used for two samples when concentration was not detected above LOQ
[d]: LOQ is used as minimum for two samples when concentration was not detected above LOQ

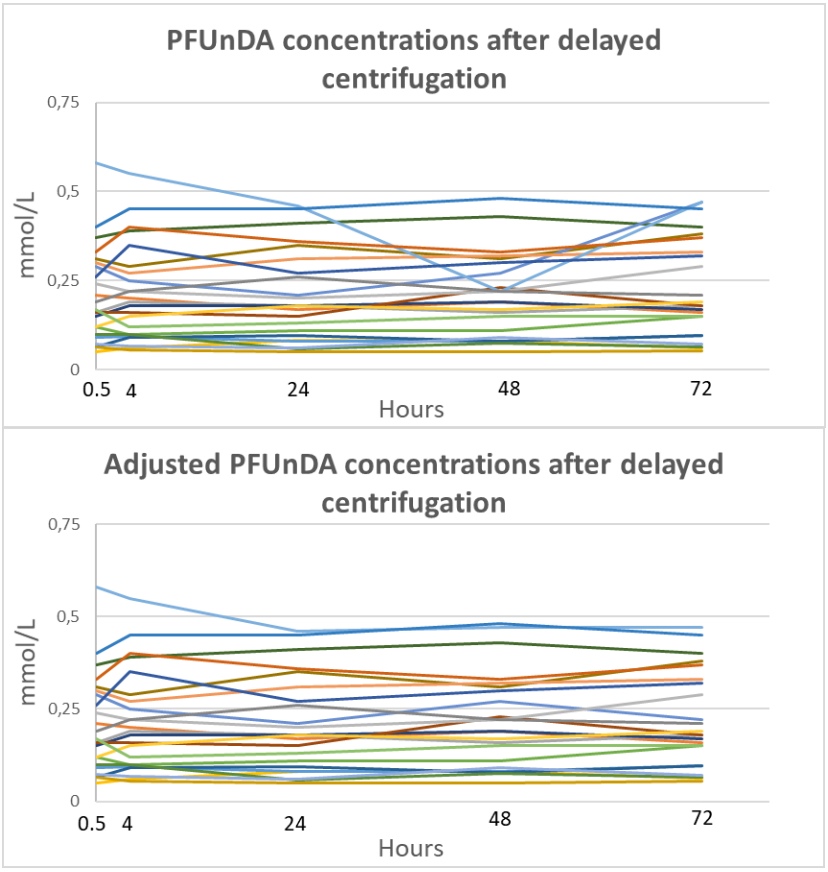

Figure 17: Reported and adjusted PFUnDA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 30: Reported and adjusted PFUnDA concentrations after delayed centrifugation: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	23	0.209	0.132	0.050[c]	0.098	0.170	0.300	0.58	0.53[d]
4 h	23	0.215	0.136	0.055	0.099	0.190	0.290	0.55	0.505
24 h	22	0.209	0.128	0.050[c]	0.095	0.180	0.310	0.46	0.41[d]
48 h [a]	22	0.207	0.116	0.050[c]	0.091	0.190	0.300	0.48	0.43[d]
48 h [b]	22	0.218	0.129	0.050[c]	0.091	0.190	0.310	0.48	0.43[d]
72 h [a]	23	0.231	0.140	0.054	0.097	0.180	0.370	0.47	0.426
72 h [b]	23	0.220	0.130	0.054	0.097	0.180	0.330	0.47	0.426

a: Original concentrations
b: Adjusted concentrations
[c]: LOQ is used for one or more samples when concentration was not detected above LOQ
[d]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

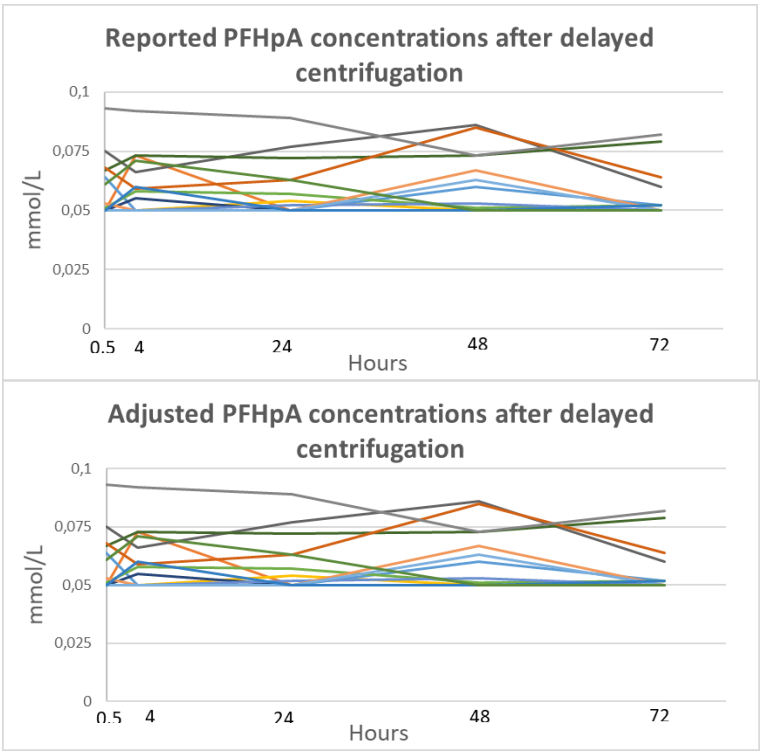

Figure 18: Reported and adjusted PFHpA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 31: Reported and adjusted PFHpA concentrations after delayed centrifugation: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h[a]	9	0.0602	0.0131	0.050[c]	0.050	0.0530	0.0675	0.093	0.043[d]
0.5 h[b]	9	0.0594	0.0129	0.050[c]	0.050	0.0520	0.0673	0.093	0.043[d]
4 h [a]	9	0.0621	0.0126	0.050[c]	0.050	0.0590	0.0720	0.092	0.042[d]
4 h [b]	9	0.0612	0.0125	0.050[c]	0.050	0.0585	0.0715	0.092	0.042[d]
24 h [a]	9	0.0598	0.0126	0.050[c]	0.050	0.0540	0.0675	0.089	0.039[d]
24 h [b]	8	0.0591	0.0124	0.050[c]	0.050	0.0530	0.0653	0.089	0.039[d]
48 h [a]	8	0.0614	0.0138	0.050[c]	0.050	0.0530	0.0730	0.086	0.036[d]
48 h [b]	9	0.0615	0.0133	0.050[c]	0.050	0.0565	0.0730	0.086	0.036[d]
72 h [a]	8	0.0580	0.0112	0.050[c]	0.050	0.0520	0.0635	0.082	0.032[d]
72 h [b]	7	0.0565	0.0110	0.050[c]	0.050	0.0510	0.0610	0.082	0.032[d]

a: Original concentrations
b: Adjusted concentrations
[c]: LOQ is used for one or more samples when concentration was not detected above LOQ
[d]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

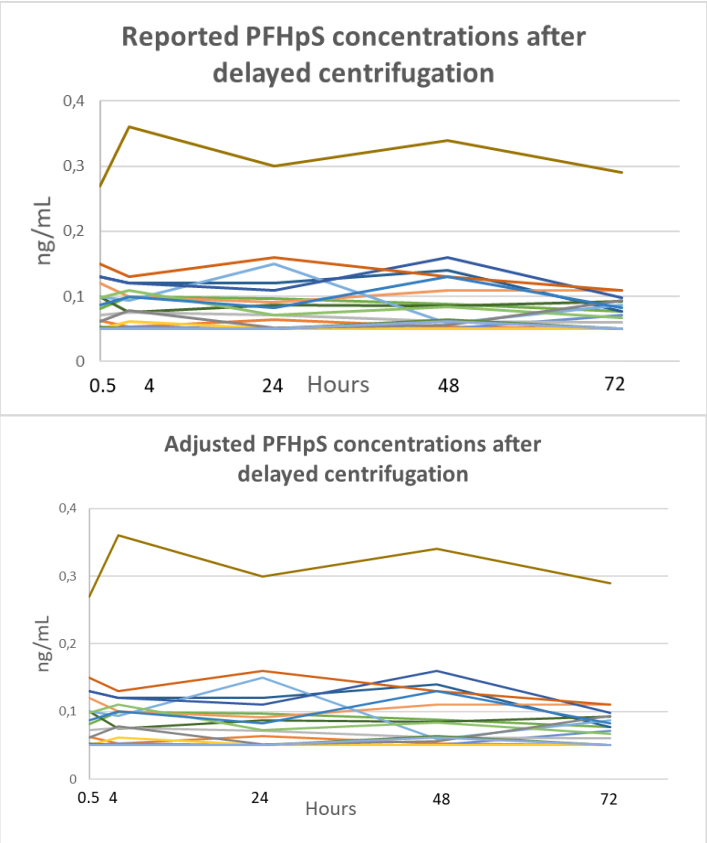

Figure 19: Reported and adjusted PFHpS concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 32: Reported and adjusted PFHpS concentrations after delayed centrifugation: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	14	0.0979	0.0545	0.050[c]	0.057	0.087	0.125	0.27	0.22[d]
4 h	16	0.1020	0.0716	0.050[c]	0.057	0.094	0.115	0.36	0.31[d]
24 h	14	0.0975	0.0624	0.050[c]	0.051	0.083	0.115	0.30	0.25[d]
48 h [a]	15	0.1020	0.0705	0.050[c]	0.0585	0.084	0.130	0.34	0.29[d]
48 h [b]	15	0.1010	0.0709	0.050[c]	0.0575	0.084	0.130	0.34	0.29[d]
72 h [a]	13	0.0885	0.0559	0.050[c]	0.0545	0.077	0.096	0.29	0.24[d]
72 h [b]	13	0.0892	0.0556	0.050[c]	0.055	0.077	0.096	0.29	0.24[d]

a: Original concentrations
b: Adjusted concentrations
[c]: LOQ is used for one or more samples when concentration was not detected above LOQ
[d]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

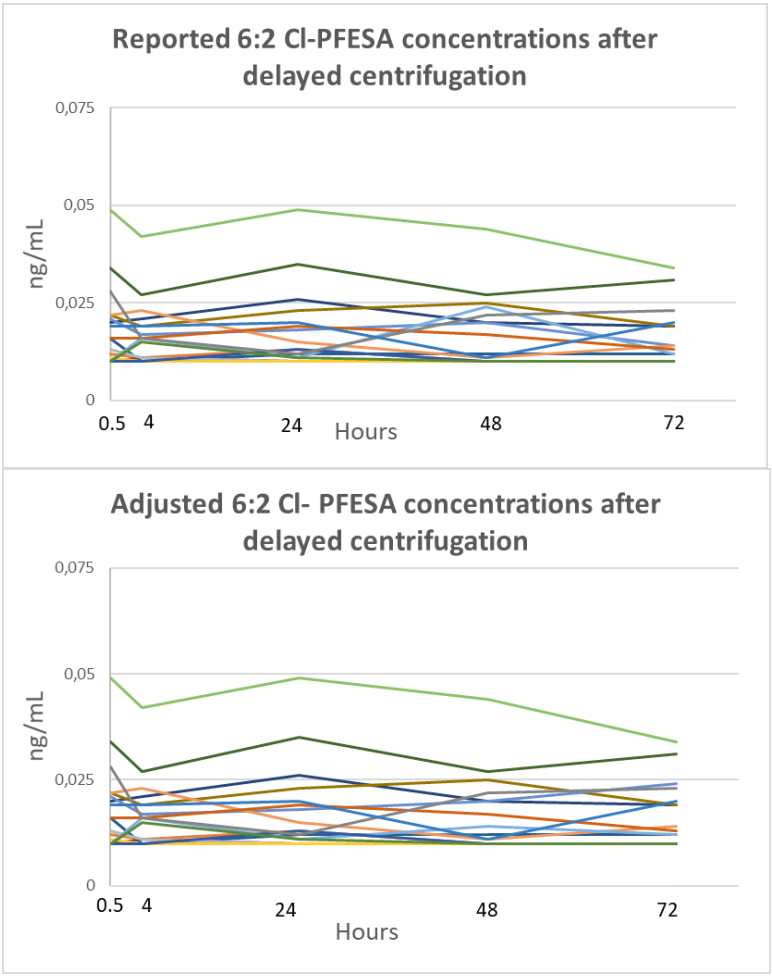

Figure 20: Reported and adjusted 6:2 Cl-PFESA concentrations in EDTA-plasma after delayed centrifugation of blood.

Table 33: Reported and adjusted 6:2 Cl-PFESA concentrations after delayed centrifugation: Descriptive values.

Delay	No. of samples above LOQ	Mean, ng/mL	Std.dev, ng/mL	Min., ng/mL	Q1, ng/mL	Median, ng/mL	Q3, ng/mL	Max., ng/mL	Range, ng/mL
0.5 h	14	0.0190	0.0104	0.010[c]	0.0105	0.016	0.0220	0.049	0.039[d]
4 h	15	0.0173	0.0081	0.010[c]	0.0110	0.016	0.0200	0.042	0.032[d]
24 h	15	0.0181	0.0105	0.010[c]	0.0110	0.013	0.0215	0.049	0.039[d]
48 h [a]	15	0.0172	0.0093	0.010[c]	0.0100	0.012	0.0230	0.044	0.034[d]
48 h [b]	15	0.0166	0.0091	0.010[c]	0.0100	0.012	0.0210	0.044	0.034[d]
72 h [a]	14	0.0159	0.0075	0.010[c]	0.0100	0.013	0.0195	0.034	0.024[d]
72 h [b]	14	0.0165	0.0077	0.010[c]	0.0100	0.013	0.0215	0.034	0.024[d]

a: Original concentrations
b: Adjusted concentrations
[c]: LOQ is used for one or more samples when concentration was not detected above LOQ
[d]: LOQ is used as minimum for one or more samples when concentration was not detected above LOQ

Table 34: Percentage differences in mean concentrations for PFAS in EDTA-plasma after delayed centrifugation

Analyte	4 vs 0.5 h	24 vs 0.5 h	48 vs 0.5 h	72 vs 0.5 h	24 vs 4 h	48 vs 4 h	72 vs 4 h	48 vs 24 h	72 vs 24 h	72 vs 48 h
PFOA	4,8	-5,4	-7,2	-2,8	-9,8	-11,4	-7,3	-1,8	2,8	4,7
PFNA	-1,6	-4,1	-9,4	-4,4	-2,5	-7,9	-2,9	-5,5	-0,4	5,5
PFHxS	-6,6	-3,4	-0,4	-5,7	3,4	6,7	1,0	3,1	-2,4	-5,4
PFOS	-8,1	-6,9	-5,9	-1,2	1,3	2,4	7,5	1,1	6,1	5,0
PFDA	3,1	-1,5	9,2	6,2	-4,5	6,0	3,0	10,9	7,8	-2,8
PFUnDA	2,9	0,0	4,3	5,3	-2,8	1,4	2,3	4,3	5,3	0,9
PFHpA	3,0	-0,5	3,5	-4,9	-3,4	0,5	-7,7	4,1	-4,4	-8,1
PFDoDA	4,2	-0,4	3,2	-8,9	-4,4	-1,0	-12,5	3,6	-8,5	-11,7
6:2 Cl-PFESA	-8,9	-4,7	-12,6	-13,2	4,6	-4,0	-4,6	-8,3	-8,8	-0,6

Investigation of the effect of delayed separation of blood after 0.5, 4, 24, 48 and 72 h on PFAS concentrations in EDTA-plasma revealed that only PFDA had statistically significant differences between the different timepoints using both the reported and adjusted concentrations. In addition, 6:2 Cl-PFESA and PFOS had statistically significant differences if the reported concentrations were used in the analysis.

6:2 CL-PFESA tended to decrease with increased delay. When comparing two and two timepoints and adjusting for multiple testing, the timepoints with the biggest difference, 0.5 and 72 h with a 13.2 % decrease in concentrations, has a statistically significant difference, p=0.037, but not any other timepoints. For the adjusted data, there was still a tendency to decreased concentrations for 48 and 72 h, but there was not a statistically significant difference between timepoints, $\chi2(4) = 7.614$, p=0.107.

For PFDA, there was a statistically significant difference between at least two timepoints for both the reported and adjusted concentrations, $\chi2(4) = 13.170$, p=0.010 and $\chi2(4) = 14.188$, p=0.007, respectively. For the reported concentrations, the biggest differences were between 24 and 48 h where the concentrations have increased with 10.9%. However, the differences in concentrations were not statistically significant when comparing two and two groups and adjusting for multiple testing, p=0.342. Using the adjusted concentrations,

there was not a statistically significant difference between any two timepoints, not either between the timepoints with the biggest differences 24 and 48 h, p=0.074.

The PFOS concentrations decreased between 0.5 and 4 h delayed separation, and then increased to approx. the original concentration after the 72-h delay. Comparison of two and two timepoints and adjusting for multiple testing revealed that for the reported concentrations, there were not any statistically significant differences, p=0.065.

4 Discussion

It is critical for clinical investigations but also for medical research that high quality biological samples are used for analysis. To obtain reproducible results, biological material which are fit for purpose must be used.

The intention of this study was to give more insight on which effect delayed centrifugation of blood and delayed freezing of plasma have on specific components in EDTA-plasma. The design was developed to mimic existing and possible processing procedures in the NIPH biobank for remote collection of samples and shipment to a central laboratory. The selection of analytes was based on the interest in Human Environmental Biobank and MoBa and the availability of analysis in EDTA-plasma.

Unfortunately, a likely sample switch was detected in the delayed centrifugation part of the project. This was discovered when looking at the data, and visualization and statistical analysis of both the reported and adjusted or corrected data have been presented under results. For most analytes, the switch has a high probability when looking at the concentrations. The samples that most likely have been switched, have also been handled at the same days and at approx. the same time before freezing. The impact of the possible error in the sample handling is minor for most of the analytes as only one sample at 48 h is switched with one at 72 h. However, this contribute to a small difference in mean and median concentrations, and in this thesis, I have given the closest attention to the adjusted results.

4.1 Evaluation of results, PFAS

To my knowledge, this is the first time the effect of storage of EDTA-plasma at room temperature before freezing and delayed separation of blood on PFAS levels in plasma are reported for analysis using a column switching liquid chromatography method coupled to a triple quadruple mass spectrometer.

PFAS are used in numerous of products, and traces can also be found in labware containing Teflon because they are used as one of the starting products for Teflon production. Thus, for the PFAS which are stable in this quality study, there is not increased contamination from the labware.

In this study, I suggest that delayed freezing of EDTA-plasma has no significant impact on most PFAS concentrations. However, it should be taken into consideration that for some of the analytes, i.e PFHpA, PFDoDA, PFHpS and 6:2 Cl-PFESA, the number of samples with concentrations above the LOQ is low and the power of the study weak. This is also the case for PFTrDA where only 24% of the samples had concentrations above LOQ and which, according to the non-parametric Friedman test, show statistically significant differences between at least two timepoints.

For the other analyte with a statistically significant difference, PFNA, the concentrations increased from 0 to 4 h and then decreased. According to the Friedman test, there was a statistically significant difference between at least two timepoints, but post-hoc Wilcoxon analysis with Bonferroni correction to adjust for multiple testing to compare two and two timepoints revealed no significant difference. When comparing two and two groups with Wilcoxon, with high numbers of groups, there is a possibility to get to groups that are significantly different even if the null hypothesis is true. The Bonferroni correction takes this into account. Nevertheless, there are discussions about whether the Bonferroni correction is too conservative for more than 3-4 groups since p-values or significance levels are adjusted with the number of comparisons (48,49). Thus, this can lead to changes being statistically significant when using the Friedman test and not when comparing two and two groups, especially when the p-values are close to 0.05 in both analyses like they are in this project.

When it comes to delayed separation of blood, our analysis revealed that the plasma levels of PFOA, PFNA, PFhXS, PFUnDA, PFHpA and PFHpS did not change significantly with delayed separation. However, regarding PFHpA, it was only measured above LOQ in 34% of the 120 samples belonging to 13 participants using the reported results and 14 participants using the adjusted results. Hence, the power of the statistics is weak.

For PFOS and PFDA, the same occurred as for PFNA after delayed freezing. There were statistically significant differences between at least two timepoints for separation of plasma, but post-hoc analysis to compare two and two timepoints revealed no statistically significant differences.

4.2 Evaluation of results – sodium, cholesterol and triglycerides

Triglycerides and cholesterol are among the most frequently investigated compounds when it comes to stability in serum and plasma (25). Previously, others like Clark et al. and Hankinson et al. have suggested that cholesterol and triglycerides are stable for 7 and 3 days, respectively, in whole blood before centrifugation or that they only have non-significant changes (20,46). For triglycerides, our findings correspond to what was previously published. However, in our study the cholesterol concentrations increased with increased delay in centrifugation from 0.5 to 24 h where the concentration seems to be stabilized. Nevertheless, Clark et al. have presented that the cholesterol concentrations change with less than 4 % after 7 days of delayed separation of blood, and after 3 days at 21°C, the change from immediately separated samples are 0.6% using a method with CV of 2% (20). The change in our project is 3.3 % using a method with a CV of 3%. Hence, when compared to the results of Clark et al., we experienced a bigger change. There are several possible reasons for our study suggesting a statistically significant change compared to other studies. It could be the power of the studies, the difference in analytical methods used or the difference in sample processing when it comes to treatment between separation of plasma and the analysis of the samples. Clark et al. included 12 participants in their study, while we had 24 due to our power estimations. When it comes to sample processing, the two studies had similar designs and are performed at approximately the same temperature, but Clark et al. covered the samples with aluminum foil while ours stood uncovered on the bench and thus, exposed to light. However, light exposure does not necessarily alter triglycerides. There could also be un-reported differences like sample hemolysis, thawing method and time before the analysis.

Even if we have found a statistically significant difference, it should be considered what impact the change may have for medical outcome and how the difference will impact the studies using the biobank material. It is difficult to set a universal limit for what change is acceptable for analytes and not. In this study, the participants are considered healthy, and the analyte concentrations are mostly within the reference values. Therefore, we do not know whether samples with considerably higher or lower concentrations will behave similarly.

When it comes to delayed freezing of plasma, our result that cholesterol is stable for the 72 hours that were studied, is in line with the product sheet for the analytical method used (26).

Previously, Taylor et al. have suggested that triglycerides in plasma are stable for up to 7 days at room temperature (18) and the product sheet for the analytical method suggests that samples can be stored in room temperature for up to two days (31). In our study, the concentrations at 0 hours stood out compared to the other timepoints, and there is a statistically significant difference between 0 and 48 hours. There are only a few studies that have investigated the effect of storage at room temperature on triglyceride concentrations. Even if our findings of changes after 24 hours are supported in the literature, caution should be taken to ensure sufficient quality for triglyceride analysis already after 24 h.

In stability studies, sodium can be used as an indicator of volume changes in plasma or serum. Oddoze et al. have reported that sodium is stable for 24 h at room temperature before processing different types of tubes (47). In our study of effects of delayed centrifugation, there is a statistically significant increase in sodium levels between samples separated after 0.5 h compared to 4, 24, 48 and 72 h. Hence, the two samples with elevated sodium concentrations at 24 h, which were treated slightly different from the rest of the samples, did not seem to have critical impact on these results. Nevertheless, the samples at 0.5 h are processed in the same manner as the samples at the other timepoints, and there is no obvious reason in the treatment for these samples to have lower concentrations.

Our investigation of the impact of delayed freezing of plasma samples showed that there was a statistically significant difference in sodium concentrations at the different timepoints, but there were no significant differences when comparing two and two groups. Previously, it was reported that sodium is stable for up to 56 h (18). In our study, the concentrations seemed to increase with increased delay, but the differences were quite small with less than 1% change from 0 to 72 h.

4.3 Sample integrity studies

To maintain sample integrity and to provide scientists with biological material that is fit for the purpose, sample quality must be maintained through the lifetime of the material. The

present study has investigated effects of delayed separation of blood and delayed freezing of EDTA-plasma on a small number of analytes but to ensure extensive use of the biological material, more analytes should be investigated. To ensure sample integrity throughout the lifetime of the biological material, knowledge about the effect of other parts of sample handling must exist, i.e. sample collection, long-time storage, and retrieval.

Worldwide numerous of integrity studies of biological material are run and published, adding small parts to pre-analytical knowledge. The biobank of the Norwegian Institute of Health has run and are running a few studies, and there are plans for further studies. Short information of blood sample/ blood derivative projects is presented in table 35.

Table 35: Overview of blood derivative sample integrity projects in The NIPH biobank

Biospecimen	Process	Number of participants	Analytes	Project status
Plasma (EDTA)	Long-time storage, -80 °C	Pooled plasma from 40 participants	Sodium Cholesterol Triglycerides AST Vitamin E	Started in 2003, ongoing
Plasma (EDTA)	Freeze-thaw – up to 30 cycles	Pooled plasma from 40 participants	Sodium Cholesterol Triglycerides AST Vitamin E Free fatty acids	Finished Published (50)
Serum	Long-time storage, three different environments at -80 °C	32	Sodium Potassium Cholesterol Triglycerides Ferritin Folate AST ALT LD CK TSH fT4 fT3 Protein total C-peptide	Started in 2018, ongoing
PBMC	Long-time storage, -150°C	To be decided	Portion of living cells Viability	Under development

4.4 Conclusion and recommendations for future collections

Cost-efficient, remote collection of biological material and subsequent transfer to a central biobank in public health surveys or other population studies can be challenging when it comes to maintain sample quality and to provide samples that have sufficient quality for the intended analysis.

In summary, this study has shown that most of the analytes we investigated in EDTA-plasma are stable even after 72 h of delayed freezing after separation from blood and after 72 h of delayed separation of blood. However, some analytes like cholesterol, sodium and PFNA had statistically significant differences between at least two timepoints after delayed centrifugation of blood. Also, PFTrDA which was only detected in 24% of the samples had a tendency to increase with increasing delay, and for PFDoDA there are twice as many samples with concentrations above LOQ at 0 h compared to samples at 4 h and four times as many at 0 h than at 72 h.

Delayed freezing of EDTA-plasma up to 72 h resulted in statistically significant differences in sodium, triglycerides and PFDA. Also, 6:2 Cl-PFESA which was not detected in all samples, tended to have different levels of the analytes, but the power is weaker.

Thus, I have shown that for some analytes, delayed centrifugation and delayed freezing of plasma up to 72 h does not change the analyte level or it changes non-statistically. This means that collection methods with shipment to a central laboratory can be a possible solution if the stable analytes are going to be investigated or that samples in a central laboratory can stay over the weekend before analyzed. Preferably, a pilot study or validation of the processing method according to ISO 20387: 2018 should be performed before the collection starts to ensure samples with sufficient quality to be fit for the intended purpose for known analytes.

Nevertheless, in many biobanks, it is not known which analysis the samples will be used for at time of sample collection. Therefore, a collection method embracing high quality samples, which are fit for as many analyses as possible, should be chosen. However, when biological material is requested for analysis, the biobank should ensure that the samples are fit for that particular method prior to potential distribution. This should be done through

collection of existing documentation or experiments like this study. Preferably, biobanks should have standard procedures for performing this kind of stability studies mimicking their own collection and processing methods and offer available test-samples which can be analyzed for specific analytes before distribution of actual biobank samples to projects.

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Appendix A– Biomarker analysis results, delayed freezing of EDTA-plasma

Table A1: Sodium concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (mmol/L)	4 h (mmol/L)	24 h (mmol/L)	48 h (mmol/L)	72 h (mmol/L)
1	140	142	141	141	142
2	140	139	140	140	141
3	140	142	142	141	142
4	145	144	144	144	144
5	141	141	142	143	141
6	141	141	140	140	142
7	140	139	140	140	141
8	140	141	141	139	140
9	138	138	140	141	139
10	140	139	138	139	141
11	141	138	141	141	141
12	137	139	137	138	139
13	140	137	137	136	137
14	137	139	138[a]	137	139
15	142	141	142	140	142
16	139	139	141	142	141
17	138	140	139	140	140
18	136	136	139	136	136
19	140	141	141	141	143
20	136	136	140	137	138
21	138	138	138	137	138[a]
22	141	141	142	141	141
23	140	141	141	141	141
24	139	139	139	138	139

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.

Table A2: Cholesterol concentrations after delayed freezing of plasma

ID/hours	0 h (mmol/L)	4 h (mmol/L)	24 h (mmol/L)	48 h (mmol/L)	72 h (mmol/L)
1	6,6	6,8	6.8	6.5	6.5
2	5.7	5.8	5.8	5.7	5.6
3	6.3	6.4	6.5	6.2	6.3
4	5.6	5.5	5.5	5.4	5.4
5	5.1	5.1	5.1	5.1	5.1
6	5.4	5.3	5.3	5.4	5.4
7	5.2	5.2	5.3	5.3	5.2
8	5.4	5.4	5.4	5.6	5.4
9	5.9	6.0	6.0	5.9	6.0
10	5.3	5.3	5.3	5.4	5.3
11	5.0	5.1	5.1	5.1	5.3
12	7.3	7.3	7.3	7.4	7.2

13	5.4	5.4	5.5	5.2	5.5
14	3.6	3.6	3.6[a]	3.7	3.8
15	5.6	5.6	5.6	5.6	5.6
16	4.7	4.8	4.9	4.8	4.8
17	6.1	6.3	6.1	6.3	6.2
18	4.8	4.7	4.8	4.8	4.8
19	5.6	5.6	5.5	5.6	5.5
20	3.9	3.9	4.0	3.9	3.9
21	7.1	7.1	7.2	7.1	6.9[a]
22	6.1	6.2	6.2	6.2	6.2
23	5.2	5.2	5.3	5.3	5.3
24	5.8	5.9	5.9	5.9	6.0

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.

Table A3: Component concentrations after delayed freezing of plasma

ID/hours	0 (mmol/L)	4 (mmol/L)	24 (mmol/L)	48 (mmol/L)	72 (mmol/L)
1	1.5	1.5	1.5	1.5	1.5
2	2.1	2.1	2.1	2.1	2.1
3	2.3	2.3	2.3	2.3	2.3
4	1.4	1.4	1.4	1.4	1.4
5	1.0	1.0	1.0	1.0	1.0
6	0.6	0.6	0.6	0.6	0.6
7	0.4	0.5	0.5	0.5	0.4
8	0.7	0.8	0.8	0.8	0.8
9	0.8	0.9	0.9	0.9	0.9
10	0.9	0.9	0.9	0.9	0.9
11	0.6	0.6	0.6	0.6	0.6
12	3.2	3.2	3.2	3.3	3.2
13	0.9	1.0	1.0	1.0	1.0
14	0.5	0.5	0.5[a]	0.5	0.5
15	0.6	0.6	0.6	0.6	0.6
16	2.8	2.8	2.8	2.8	2.8
17	1.4	1.4	1.4	1.4	1.4
18	1.0	1.0	1.0	1.0	1.0
19	1.4	1.4	1.4	1.4	1.4
20	0.9	0.9	0.9	0.9	0.9
21	1.6	1.6	1.6	1.6	1.6[a]
22	1.7	1.8	1.8	1.8	1.8
23	2.3	2.4	2.4	2.4	2.4
24	0.7	0.8	0.8	0.8	0.8

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.

Table A4: PFOA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.82	1.0	0.92	0.90	0.83
2	0.54	0.70	0.45	0.61	0.51
3	1.1	1.3	1.2	1.3	1.3
4	1.1	1.3	1.2	1.3	1.3
5	1.5	1.4	1.6	1.6	1.9
6	0.58	0.59	0.56	0.45	0.62
7	0.25	0.20	0.18	0.20	0.21
8	1.4	1.4	1.5	1.6	1.4
9	0.77	0.92	0.73	0.88	0.89
10	1.4	1.7	1.4	1.3	1.5
11	0.44	0.51	0.48	0.47	0.62
12	0.96	0.95	0.71	0.77	0.9
13	1.6	1.8	1.5	1.6	1.1
14	1.0	1.1	1.0	0.92	1.1
15	0.37	0.46	0.38	0.35	0.28
16	0.98	1.2	0.97	1.0	1.2
17	2.4	2.0	2.7	2.6	2.2
18	1.3	1.4	1.4	1.4	1.3
19	1.5	1.5	1.4	1.2	1.1
20	0.64	0.73	0.58	0.58	0.85
21	0.52	0.54	0.54	0.69	0.48
22	1.2	1.1	1.3	1.1	1.3
23	0.82	0.54	0.61	0.73	0.85
24	1.4	1.4	1.4	1.5	1.5

Table A5: PFNA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72h (ng/mL)
1	0.51	0.62	0.56	0.44	0.51
2	0.33	0.53	0.53	0.52	0.53
3	0.48	0.49	0.55	0.38	0.42
4	0.59	0.57	0.61	0.54	0.40
5	0.81	1.1	1.0	0.81	0.77
6	0.18	0.12	0.18	0.13	0.13
7	0.15	0.17	0.18	0.19	0.17
8	0.71	0.96	0.72	0.78	0.83
9	0.44	0.45	0.55	0.37	0.45
10	0.87	0.83	0.68	0.94	0.69
11	0.32	0.32	0.26	0.27	0.33
12	0.50	0.34	0.30	0.36	0.41
13	0.35	0.32	0.40	0.49	0.38
14	0.19	0.17	0.20	0.17	0.14
15	0.28	0.33	0.28	0.25	0.40
16	0.35	0.34	0.35	0.34	0.34

17	1.1	1.1	0.97	0.87	1.0
18	0.40	0.36	0.35	0.33	0.32
19	0.56	0.53	0.49	0.55	0.49
20	0.20	0.16	0.15	0.14	0.11
21	0.12	0.12	0.08	0.12	0.11
22	0.60	0.63	0.61	0.47	0.46
23	0.41	0.42	0.34	0.40	0.29
24	1.0	0.79	0.72	0.75	0.83

Table A6: PFHxS concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.32	0.35	0.28	0.32	0.36
2	0.18	0.26	0.18	0.26	0.24
3	0.34	0.41	0.36	0.35	0.36
4	0.40	0.41	0.44	0.39	0.39
5	1.5	1.0	1.3	1.3	1.4
6	0.11	0.094	0.12	0.11	0.12
7	0.12	0.097	0.14	0.13	0.12
8	0.40	0.50	0.60	0.50	0.47
9	0.44	0.45	0.33	0.40	0.45
10	0.65	0.66	0.73	0.65	0.52
11	0.23	0.23	0.22	0.23	0.22
12	1.1	0.79	0.87	0.92	0.76
13	0.50	0.33	0.47	0.35	0.30
14	0.37	0.29	0.42	0.40	0.38
15	0.12	0.13	0.17	0.084	0.14
16	0.73	0.78	0.89	0.74	0.65
17	0.86	0.90	0.99	1.1	0.95
18	0.38	0.40	0.49	0.48	0.43
19	0.71	0.52	0.50	0.59	0.56
20	0.22	0.32	0.24	0.27	0.14
21	0.14	0.13	0.15	0.097	0.14
22	0.29	0.37	0.41	0.31	0.37
23	1.4	1.1	1.0	1.0	0.96
24	0.63	0.57	0.61	0.51	0.76

Table A7: PFOS concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	5.0	5.5	6.2	5.4	6.2
2	1.5	1.7	1.7	1.6	1.8
3	1.8	2.2	1.8	1.8	2.2
4	3.3	2.8	2.5	3.0	2.7
5	6.4	5.8	6.2	6.1	5.2
6	0.39	0.35	0.36	0.38	0.55

7	0.62	0.94	1.0	1.1	0.77
8	4.2	3.5	4.1	3.7	3.7
9	1.8	1.4	1.4	1.7	1.4
10	7.7	5.9	5.3	6.7	7.2
11	1.4	1.5	1.5	1.3	1.2
12	4.0	4.0	4.7	3.8	5.3
13	3.2	2.8	2.8	3.1	2.7
14	1.7	1.5	1.5	1.4	1.4
15	1.7	1.9	1.5	1.6	1.8
16	3.7	3.6	3.4	4.0	3.2
17	5.7	5.9	6.6	6.1	6.5
18	2.9	3.1	3.0	2.6	3.2
19	4.4	3.8	3.6	4.3	3.5
20	1.0	0.68	0.72	0.85	0.69
21	0.38	0.33	0.37	0.56	0.4
22	4.5	4.0	4.9	4.2	3.7
23	1.7	1.3	1.7	1.5	1.5
24	5.0	5.5	6.2	5.4	6.2

Table A8: PFDA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.20	0.19	0.18	0.24	0.25
2	0.17	0.16	0.15	0.11	0.15
3	0.13	0.15	0.13	0.21	0.15
4	0.11	0.11	0.11	0.10	0.096
5	0.40	0.44	0.46	0.44	0.40
6		0.077	0.073	0.066	0.068
7	0.087	0.087	0.11	0.09	0.11
8	0.22	0.21	0.31	0.25	0.25
9	0.16	0.14	0.10	0.14	0.16
10	0.33	0.37	0.34	0.37	0.34
11	0.11	0.16	0.12	0.07	0.16
12	0.12	0.15	0.1	0.11	0.13
13	0.13	0.11	0.091	0.16	0.13
14	0.068	0.054	0.073	0.063	0.053
15	0.16	0.20	0.14	0.15	0.17
16	0.084	0.087	0.07	0.073	0.074
17	0.37	0.35	0.32	0.32	0.43
18	0.12	0.12	0.058		0.084
19	0.24	0.18	0.19	0.18	0.15
20					
21					
22	0.20	0.21	0.23	0.23	0.20
23	0.097		0.07	0.093	0.11
24	0.35	0.41	0.35	0.42	0.33

Table A9: PFUnDA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.24	0.31	0.25	0.29	0.18
2	0.15	0.18	0.16	0.17	0.19
3		0.061	0.05	0.05	
4	0.081	0.07		0.061	0.064
5	0.59	0.51	0.51	0.40	0.49
6					
7	0.10	0.098	0.075	0.12	0.13
8	0.32	0.36	0.39	0.32	0.37
9	0.11	0.092	0.072	0.14	0.1
10	0.35	0.44	0.40	0.34	0.44
11	0.19	0.23	0.18	0.15	0.19
12	0.079	0.093	0.071	0.059	0.09
13	0.089	0.073	0.065	0.065	0.07
14					
15	0.16	0.19	0.22	0.15	0.17
16					
17	0.43	0.58	0.53	0.43	0.53
18		0.057	0.060	0.055	0.059
19	0.20	0.21	0.20	0.27	0.18
20					
21					
22	0.28	0.27	0.28	0.22	0.21
23		0.057			0.056
24	0.30	0.28	0.38	0.29	0.29

Table A10: PFHpA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1					
2	0.056				0.058
3	0.056	0.053	0.059	0.051	0.065
4	0.068	0.056	0.061	0.063	0.064
5	0.07	0.082	0.072	0.070	0.083
6	0.063	0.073	0.055	0.052	0.053
7	0.051	0.050			
8	0.096	0.120	0.110	0.100	0.088
9		0.067			
10			0.050		0.056
11			0.051	0.052	
12		0.056			0.054
13		0.069	0.051	0.071	
14					
15					
16					

17	0.056	0.055	0.052		0.060
18					
19	0.059	0.051			
20					0.067
21		0.062		0.058	0.071
22	0.107	0.114	0.100	0.078	0.103
23	0.063	0.063			
24					

Table A11: PFDoDA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.050				
2	0.050			0.053	
3					
4					
5	0.077	0.076	0.075	0.057	0.065
6					
7			0.052		
8	0.058	0.074	0.059	0.057	
9					
10	0.056	0.055	0.059		0.063
11	0.060	0.053			
12					
13					
14					
15	0.051				
16	0.050				
17					
18					
19					
20					
21					
22					
23					
24					

Table A12: PFTrDA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.051	0.063		0.073	
2			0.062	0.068	0.065
3					
4					
5	0.088	0.067	0.100	0.092	0.100
6					
7				0.050	0.053

8	0.075	0.07	0.076	0.087	0.097
9					
10	0.066	0.054	0.062	0.062	0.075
11		0.053	0.055	0.067	
12					
13					
14					
15					
16					
17	0.050				
18					
19					
20					
21					
22	0.065				
23					
24				0.054	

Table A13: PFHpS concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.067	0.062	0.056	0.084	0.069
2	0.053	0.061		0.054	
3	0.059	0.068	0.076	0.058	0.066
4	0.06	0.077	0.099	0.072	0.073
5	0.14	0.13	0.10	0.12	0.13
6					
7					
8	0.11	0.083	0.10	0.11	0.088
9	0.055	0.061		0.057	0.05
10	0.20	0.18	0.21	0.21	0.17
11			0.063	0.051	
12	0.17	0.14	0.14	0.15	0.15
13					0.057
14					
15					
16	0.096	0.095	0.11	0.10	0.092
17	0.086	0.091	0.084	0.098	0.13
18	0.051			0.054	
19		0.053		0.069	
20					
21					
22					
23					
24	0.12	0.053	0.11	0.10	0.10

Table A14: 6:2 Cl-PFESA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.015	0.016	0.018	0.022	0.015
2					
3					
4	0.011				
5	0.014	0.026	0.017	0.026	0.022
6					
7	0.011	0.014	0.012	0.01	
8	0.04	0.033	0.039	0.03	0.03
9	0.011				
10	0.02	0.022	0.017	0.018	0.016
11					
12	0.012	0.013		0.011	0.012
13				0.018	
14					
15	0.028	0.012			
16		0.012			
17	0.011	0.017			
18					0.013
19	0.035		0.011	0.044	0.01
20					
21					
22					
23					
24				0.019	

Appendix B– Biomarker analysis results, delayed centrifugation of blood and separation of EDTA-plasma

Table B1: Sodium concentrations measured after delayed centrifugation of whole blood.

ID	0.5 h (mmol/L)	4 h (mmol/L)	24 h (mmol/L)	48 h (mmol/L)	72 h (mmol/L)
1	140	141	140	141	140
2	140	142	141	141	141
3	137	138	139	138	137
4	138	138	139	138	139
5	139	140	141	140	140
6	140	140	140	138	138
7	138	140	141	142	141
8	138	139	138	138	138
9	138	139	141	142	139
10	135	136	137	135	135
11	137	139	138	138	138
12	136	138	138	138	142[b] (139)
13	137	137	147[a]	140	140
14	138	139	140	140	139
15	138	140	139	139	138
16	140	141	152[a]	139[b] (142)	141
17	137	140[c] (137)	139	140	139
18	138	140[c] (140)	139	139	140
19	139	139[c](140)	139	139	140
20	138	137[c] (139)	140	140	140
21	136	136	136	135	135
22	137	140	138	139	138
23	139	140	142	142	141
24	138	139	140	142[a]	142[a]

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.
[b]: Suspected sample mix-up between the two samples. Suggested correct value is in brackets.
[c]: Suspected sample mix-up between the four samples. Suggested correct value is in brackets.

Table B2: Cholesterol concentrations measured after delayed centrifugation of whole blood.

ID	0 h (mmol/L)	4 h (mmol/L)	24 h (mmol/L)	48 h (mmol/L)	72 h (mmol/L)
1	5.0	5.0	5.1	5.1	5.1
2	5.7	5.9	5.9	6.0	5.8
3	6.0	6.2	6.2	6.1	6.1
4	4.6	4.7	4.8	4.8	4.7
5	5.2	5.2	5.3	5.3	5.3
6	3.2	3.1	3.2	3.2	3.2
7	5.1	5.2	5.2	5.2	5.2
8	4.2	4.3	4.4	4.5	4.4
9	5.9	5.9	6.1	6.2	6.2
10	7.3	7.3	7.4	7.4	7.3

11	6.7	6.5	6.8	6.8	6.7
12	6.8	6.9	7.1	7.1	6.5[b] (7.0)
13	5.8	5.9	6.1[a]	6.0	6.2
14	5.4	5.4	5.5	5.3	5.6
15	4.1	4.1	4.1	4.2	4.2
16	6.2	6.3	6.6[a]	7.0[b] (6.5)	6.6
17	3.6	4.4[c] (3.6)	3.7	3.8	3.8
18	4.3	6.3[c] (4.4)	4.5	4.5	4.6
19	6.2	4.4[c] (6.3)	6.5	6.4	6.5
20	4.3	3.6[c] (4.4)	4.4	4.5	4.6
21	3.5	3.4	3.6	3.5	3.5
22	4.4	4.4	4.4	4.5	4.6
23	4.1	4.2	4.3	4.3	4.3
24	5.2	5.3	5.2	5.3[a]	5.4[a]

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.
[b]: Suspected sample mix-up between the two samples. Suggested correct value is in brackets.
[c]: Suspected sample mix-up between the four samples. Suggested correct value is in brackets.

Table B3: Triglyceride concentrations measured after delayed centrifugation of whole blood.

ID	0 h (mmol/L)	4 h (mmol/L)	24 h (mmol/L)	48 h (mmol/L)	72 h (mmol/L)
1	0.8	0.8	0.8	0.8	0.7
2	1.7	1.7	1.7	1.7	1.6
3	2.4	2.5	2.4	2.4	2.4
4	0.8	0.8	0.8	0.8	0.8
5	1.5	1.5	1.5	1.5	1.5
6	0.8	0.8	0.8	0.8	0.8
7	0.7	0.8	0.7	0.7	0.8
8	0.4	0.5	0.5	0.5	0.5
9	0.7	0.8	0.8	0.7	0.7
10	2.8	2.4	2.8	2.7	2.8
11	0.6	0.6	0.7	0.6	0.6
12	2.3	2.4	2.4	2.4	1.5[b] (2.4)
13	0.8	0.8	0.9[a]	0.8	0.8
14	0.8	0.8	0.9	0.8	0.9
15	1.6	1.6	1.6	1.6	1.6
16	1.5	1.4	1.5[a]	2.4[b] (1.5)	1.5
17	0.9	1.1[c] (0.9)	0.9	0.9	0.9
18	1.0	1.4[c] (1.1)	1.0	1.0	1.0
19	1.4	1.1[c] (1.4)	1.4	1.4	1.4
20	1.1	0.9[c] (1.1)	1.1	1.1	1.1
21	1.3	1.3	1.4	1.3	1.4
22	0.5	0.5	0.5	0.5	0.5
23	0.9	0.9	0.9	0.9	0.9
24	1.0	1.1	1.0	1.1[a]	1.1[a]

[a]: The sample has gone through one additional freeze-thaw cycle and has been analyzed at a different timepoint than the other samples from the participant.
[b]: Suspected sample mix-up between the two samples. Suggested correct value is in brackets.
[c]: Suspected sample mix-up between the four samples. Suggested correct value is in brackets.

Table B4: PFOA concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	1.42	1.32	0.99	1.29	1.20
2	0.47	0.68	0.57	0.49	0.6
3	0.73	0.88	0.89	0.65	0.93
4	1.46	1.58	1.27	1.56	1.33
5	1.36	1.29	1.18	1.10	1.19
6	0.44	0.46	0.49	0.5	0.54
7	0.50	0.42	0.38	0.39	0.55
8	0.53	0.54	0.57	0.58	0.64
9	1.85	1.63	1.73	1.59	2.01
10	0.88	0.91	0.74	0.81	0.73
11	1.49	1.68	1.09	1.44	1.81
12	0.96	0.98	0.97	0.63	2.27[a]
13	1.36	1.39	1.43	1.66	1.39
14	1.07	1.03	1.11	0.88	0.93
15	0.91	0.86	0.79	0.85	1.03
16	2.21	2.98	2.34	0.63[a]	1.93
17	0.71	1.03	0.77	0.92	1.00
18	1.61	2.07	1.86	1.86	1.6
19	2.51	2.50	2.00	2.10	2.01
20	1.01	0.79	1.03	1.01	1.12
21	0.67	0.71	0.71	0.64	0.56
22	1.44	1.37	1.60	1.40	1.66
23	1.33	1.28	0.99	1.38	0.86
24	0.55	0.41	0.5	0.55	0.48

[a]: Suspected sample mix-up between the two samples.

Table B5: PFNA concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.47	0.44	0.51	0.49	0.41
2	0.43	0.46	0.38	0.38	0.36
3	0.26	0.22	0.25	0.36	0.27
4	0.44	0.49	0.41	0.35	0.41
5	0.54	0.44	0.53	0.45	0.44
6	0.35	0.33	0.36	0.30	0.30
7	0.34	0.31	0.31	0.26	0.33
8	0.13	0.18	0.16	0.11	0.15
9	0.81	0.68	0.68	0.80	0.95
10	0.47	0.35	0.43	0.34	0.34
11	1.01	0.80	0.86	0.72	0.71
12	0.50	0.48	0.64	0.53	0.90[a]
13	0.60	0.70	0.62	0.70	0.83
14	0.65	0.50	0.53	0.50	0.51
15	0.25	0.33	0.23	0.25	0.3
16	1.25	1.20	1.05	0.50[a]	0.94

17	0.55	0.60	0.59	0.59	0.76
18	0.74	0.75	0.79	0.68	0.84
19	1.19	1.51	1.41	1.13	1.25
20	0.71	0.73	0.59	0.82	0.51
21	0.29	0.22	0.25	0.18	0.24
22	0.91	0.93	0.76	0.73	0.96
23	0.46	0.46	0.44	0.45	0.35
24	0.18	0.20	0.20	0.24	0.28

[a]: Suspected sample mix-up between the two samples.

Table B6: PFOS concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	4.1	2.8	3.3	3.0	3.2
2	1.5	1.5	2.1	1.6	1.9
3	2.4	2.4	2.1	2.1	2.3
4	2.0	1.6	2.0	2.5	2.1
5	4.7	4.4	4.8	4.5	5.2
6	1.2	1.4	1.7	1.4	1.3
7	1.3	1.5	1.3	1.5	1.7
8	0.4	0.46	0.46	0.46	0.46
9	11.6	10.3	12.0	10.5	13.2
10	5.0	4.1	3.7	4.2	4.1
11	4.4	3.5	3.7	4.1	4.1
12	4.1	3.1	4.1	3.8	6.6[a]
13	6.1	5.4	6.2	5.8	6.4
14	3.5	3.9	3.8	3.0	3.6
15	1.9	2.0	1.7	1.8	2.1
16	7.4	6.2	5.6	3.9[a]	6.8
17	3.8	3.5	3.4	4.0	4.1
18	7.8	7.6	7.3	7.4	6.1
19	7.0	7.1	6.6	6.7	6.7
20	4.6	4.9	4.3	5.7	5.5
21	0.7	0.79	0.67	0.77	0.82
22	7.8	6.8	6.3	6.1	6.9
23	3.1	3.1	2.6	3.0	2.4
24	0.9	0.8	0.8	1.0	1.0

[a]: Suspected sample mix-up between the two samples.

Table B7: PFDA concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.16	0.16	0.15	0.25	0.17
2	0.15	0.16	0.17	0.20	0.17
3	0.07	0.057	0.085	0.054	0.073
4	0.11	0.14	0.11	0.15	0.14
5	0.14	0.16	0.14	0.16	0.13

6	0.13	0.14	0.11	0.13	0.17
7	0.12	0.11	0.11	0.17	0.13
8			0.056	0.062	0.059
9	0.32	0.25	0.28	0.28	0.29
10	0.09	0.12	0.096	0.11	0.089
11	0.31	0.26	0.32	0.30	0.30
12	0.26	0.24	0.24	0.27	0.37[a]
13	0.3	0.26	0.25	0.29	0.28
14	0.19	0.25	0.21	0.25	0.25
15	0.12	0.16	0.15	0.14	0.21
16	0.37	0.38	0.29	0.25[a]	0.50
17	0.17	0.19	0.16	0.21	0.19
18	0.26	0.37	0.30	0.31	0.38
19	0.37	0.46	0.38	0.40	0.38
20	0.33	0.30	0.42	0.36	0.30
21	0.059			0.058	0.076
22	0.45	0.40	0.40	0.41	0.27
23	0.098	0.11	0.09	0.096	0.10
24		0.057		0.073	0.061

[a]: Suspected sample mix-up between the two samples.

Table B8: PFUnDA concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.21	0.20	0.17	0.19	0.16
2	0.16	0.19	0.18	0.16	0.18
3	0.05	0.061	0.082	0.08	0.062
4	0.09	0.093	0.081	0.081	0.097
5	0.12	0.10	0.11	0.11	0.15
6	0.15	0.18	0.18	0.19	0.17
7	0.16	0.16	0.15	0.23	0.18
8					
9	0.31	0.29	0.35	0.31	0.38
10	0.065	0.090	0.095	0.079	0.096
11	0.37	0.39	0.41	0.43	0.4
12	0.29	0.25	0.21	0.27	0.47[a]
13	0.30	0.27	0.31	0.32	0.33
14	0.24	0.22	0.2	0.22	0.29
15	0.12	0.15	0.18	0.17	0.19
16	0.58	0.55	0.46	0.22[a]	0.47
17	0.17	0.12	0.13	0.15	0.15
18	0.26	0.35	0.27	0.3	0.32
19	0.33	0.40	0.36	0.33	0.37
20	0.19	0.22	0.26	0.22	0.21
21	0.065	0.055			0.054
22	0.40	0.45	0.45	0.48	0.45
23	0.098	0.099	0.058	0.075	0.064
24	0.072	0.067	0.061	0.091	0.071

[a]: Suspected sample mix-up between the two samples.

Table B9: PFHpA concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1		0.073			
2					
3			0.054		
4	0.064			0.060	0.052
5	0.051	0.058	0.057	0.051	0.052
6		0.055			
7					
8	0.075	0.066	0.077	0.086	0.060
9					
10					
11	0.067	0.073	0.072	0.073	0.079
12	0.050		0.052	0.053	0.063[a]
13	0.053			0.067	
14					
15					
16				[a]	
17					
18					
19	0.068	0.059	0.063	0.085	0.064
20	0.093	0.092	0.089	0.073	0.082
21					
22		0.060			0.052
23	0.061	0.071	0.063		
24					

[a]: Suspected sample mix-up between the two samples.

Table B10: PFHpS concentrations measured after delayed centrifugation of whole blood.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.063	0.053	0.064	0.053	
2					
3					
4					
5	0.081	0.099	0.097	0.088	0.077
6					
7					
8					
9	0.27	0.36	0.30	0.34	0.29
10	0.13	0.12	0.12	0.14	0.077
11	0.10	0.075	0.087	0.085	0.092
12		0.053			0.059
13	0.12	0.10	0.091	0.11	0.11
14	0.072	0.076	0.071	0.062	0.06
15		0.061			
16	0.10	0.094	0.15	0.071	0.087

17	0.098	0.11	0.072	0.084	0.067
18	0.13	0.12	0.11	0.16	0.098
19	0.15	0.13	0.16	0.13	0.11
20	0.061	0.078	0.052	0.056	0.094
21					
22	0.087	0.10	0.083	0.13	0.083
23	0.053	0.05	0.05	0.064	
24				0.061	

[a]: Suspected sample mix-up between the two samples.

Table B11: 6:2 CL PFESA concentrations measured after delayed freezing of EDTA-plasma.

ID	0 h (ng/mL)	4 h (ng/mL)	24 h (ng/mL)	48 h (ng/mL)	72 h (ng/mL)
1	0.012	0.011	0.013	0.010	0.010
2					
3					
4					
5					
6	0.020	0.021	0.026	0.020	0.019
7		0.011			
8					
9	0.022	0.019	0.023	0.025	0.019
10	0.016		0.012	0.012	0.012
11	0.034	0.027	0.035	0.027	0.031
12	0.021	0.017	0.018	0.020	0.014[a]
13	0.022	0.023	0.015	0.011	0.014
14	0.013	0.011		0.010	
15	0.011				
16		0.016	0.011	0.024[a]	0.012
17	0.049	0.042	0.049	0.044	0.034
18			0.013	0.01	
19	0.016	0.016	0.019	0.017	0.013
20	0.028	0.016	0.012	0.022	0.023
21					
22	0.019	0.019	0.020	0.011	0.020
23		0.015	0.011		0.010
24	0.012	0.011	0.013	0.010	0.010

[a]: Suspected sample mix-up between the two samples.