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MASTER THESIS

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Microplastic studies in humans: evidence from feces and effects in blood

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Acknowledgment

Abstract

Background: The global increase in plastic production has been a matter of debate and a growing environmental concern the past decades. As a result, microplastic have been discovered in several environmental media and detected in the food chain. Evidence suggests that humans are ingesting microplastic through contaminated food and drink and microplastic has been discovered in human feces albeit in a limited number of individuals. Thus, a proportion number of ingested MP may be absorbed and translocated into the human blood stream. A limited number of studies have identified nanoplastic can interact with platelets and thus increase the risk of thrombosis.

Aim: Provide evidence for the occurrence of microplastic in the human gastrointestinal tract and investigate the effects of microplastic on platelet functions and interactions with leukocytes.

Methods: A digestion protocol to extract microplastic from feces was optimized from a rat feces protocol. Feces were obtained from 20 healthy volunteers. During a three-days step protocol the samples were digested to remove all the organic matter prior to FTIR at NORCE microplastic lab in Stavanger.

To evaluate the possible effect of microplastic on platelets, five concentrations (approx. 500, 250, 125, 62.5 and 31.25 μg/ml) of microplastic (sized < 25μm) were mixed with blood and tested in duplicate for 4 different polymers, respectively poly (methyl methacrylate), polyamide, polyvinyl chloride and polystyrene. Leukocytes and platelets were stained with specific antibodies and the monocyte- and neutrophile-platelets aggregates were analyzed and identified by flow cytometry.

Results: From 20 samples, 10 samples could be analyzed by FTIR. We showed the presence of microplastic particles in all the 10 fecal samples that were analyzed by the FTIR. The analysis demonstrated the presence of 3 different polymers, respectively polypropylene, polyethylene and polystyrene, where polyethylene were the dominant polymer.

Addition of microplastic in different concentrations to whole blood did not affect the formation of leukocyte-platelets aggregates in a dose-response matter, even though some of the polymers showed increased aggregation at the highest concentration.

Conclusion: Due to the limited numbers of data in both studies, the results should be interpreted as preliminary. However, we showed the presence of MP in all samples that were analyzed by

the FTIR and thus demonstrated the presence of microplastic in human feces. As for the flow cytometry analysis, we cannot conclude that microplastic did have an effect on platelet aggregation, neither for neutrophils nor monocytes, as none of the polymer showed repeatedly increase in aggregates in a dose-response manner.

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Abbreviations

μm: Micrometer

APC: Allophycocyanin

CVD: Cardiovascular disease

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

EU: European Union

FC: Flow cytometry

FDA: Food and Drug Administration

FITC: Fluorescein

FPA: Focal Plane Array

FSC: Forward scatter

FTIR: Fourier-transform infrared spectroscopy

H_2O_2: Hydrogen peroxide

HDPE: High density polyethylene

HNO_3: Nitric acid

IR: Infrared

KOH: Potassium peroxide

LDPE: Low density polyethylene

LPA: Leukocyte-platelet aggregate

MCT: Mercury cadmium telluride

mm: Millimeter

MP: Microplastic

MPA: Monocyte-platelet aggregate

nm: Nanometer

NP: Nanoplastic

PA: Polyamide

PBT: Optical fibers

PC: Polycarbonate

PE: Polyethylene

PE (fluorochrome): R-phycoerythrin

PET: Polyethylene terephthalate

PMMA: Poly (methyl methacrylate)

PNA: Platelet-neutrophils aggregate

POPs: Persistent organic pollutants

PP: Polypropylene

PPACK: Phe-Pro-Arg-chloromethylketone

PS: Polystyrene

PTFE: Polytetrafluorethylene

PUR: Polyurethane

PVC: Polyvinyl chloride

py-GC/MS: Pyrolysis-gas chromatography-mass spectrometry

QA/QC: Quality assurance and quality control

SSC: Side scatter

WHO: World Health Organization

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1   Introduction

1.1 Plastics and microplastic

The global increase in plastic production has been a matter of debate and a growing concern in the past decades (1). Since the 1930s the production of plastic has increased rapidly mainly due to the transition from coal to petroleum. The annual production of plastic has constantly increased, and in 2020 up to 367 million tons of plastics were produced globally (2, 3). Plastic polymers are being utilized in many applications and have become an essential material, due to their lightweight, inexpensively, durable structure and electrical insulation properties (4), favoring their widespread use in all areas of our everyday life. Circa 40% of the plastic production is used for food packaging, which ensures food security and protects the food from damage and contamination (5, 6). A large percentage of the plastic waste however, is not recycled and approximately 10 million tons reach the ocean every year, especially in areas where proper infrastructure and waste management is lacking (6). Even in the European Union (EU), with well-organized waste management in place, only 29.7% of the plastic is being recycled and 30.8% reach landfills. Unfortunately, the remaining plastic waste will most likely end up in the ocean due to loss during transportation, dumping or inadequate discarding (7). As a result of an enormous production and usage, a slow degradation rate combined with increased production and mismanagement over the last decades, plastic has been reported in several environmental medias (8). When plastic debris enters the waste stream it can break further down to smaller pieces, such as microplastic (MP) and nanoplastic (NP) due to weathering or aging. These fragments could become either airborne or aquatic pollutants. The degradation of plastic debris facilitates the release of other fragments attached to the surface (1, 6). The increasing plastic production includes increased material waste, which has created numerous environmental challenges. Due to this, plastic pollution has become one of the world's major environmental threats (9, 10). This global problem likely affects all ecosystems, and therefore a possible threat for entering the food chain (5). Human exposure to microplastic is likely to increase over time. If today's waste production and mismanagement continue at this rate, it is estimated that by 2050 there will be 12 000 tons of plastic waste in landfills (1).

Plastic is a commonly used term for describing several synthetic or semi-synthetic materials. Plastic usually consists of one or more polymers, in addition to several additives. The vast majority of plastics are derived from petroleum, but also from cellulose (2, 11). One of the characteristic qualities of plastic is that at some stage in the process the polymers are liquid,

making it possible for the plastic to be formed into various products (2). The properties of the polymers are often modified with the addition of different additives, such as antioxidants, flame retardants, plasticizers, pigments or softeners during the manufacturing process (2). Only in some cases, the polymers are free from additives. Consequently, the additives affect the plastic's chemical composition, properties, and costs. Plastic can be divided into two main categories: thermoplastic and thermosetting plastic. Thermoplastic does not undergo a chemical change when heated or cooled. Therefore, it can be heated, remodeled and frozen repeatedly (2). Thermoplastic includes several polymers, such as polyethylene (PE), polypropylene (PP), polyamide (PA), and polyvinyl-chloride (PVC), with PE and PP accounting for the majority (50% in 2020) of the plastic production (2, 3). Thermosetting plastic on the other hand cannot be re-melted and reformed if heated, as it will undergo chemical modification when the temperature increases, and the polymer create an insoluble three-dimensional network. Once the polymers are bound together, they cannot change structure (2, 12). This category includes polyurethane (PUR), epoxy resins, silicone, unsaturated polyester vinyl and acrylic resins (12). There are many different types of plastic polymers, all of them for different kinds of usage. Nowadays, plastics include more than twenty families of polymers among which six are known as the "big six": PP, PE, PVC, PUR, polyethylene terephthalate (PET) and polystyrene (PS), which are mainly produced from fossil fuels such as oil, gas or coal, and are designed to meet the very different needs of the end products. The "big six" represent 80% of plastic production in Europe (3). An overview of the most common plastic polymers is given in Figure *1* and Table **1**.
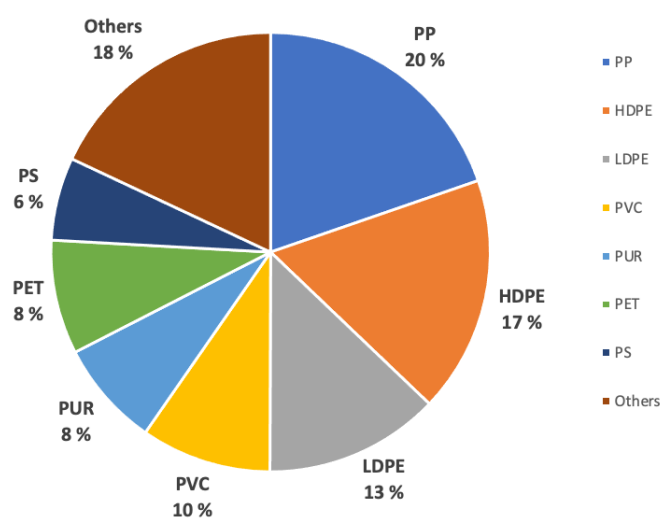
Figure 1. Plastic demand and distribution by polymer type. Data is collected from 2021 (3). Abbreviations are given in Table 1.

Table 1. Different plastic polymers and their applications. Data is from 2020 and 2021 (2, 3).

Type of polymer	Utilization
Polypropylene (PP)	Bottles, containers, ropes, chemical appliances, food packaging
Low density polyethylene (LDPE)	Reusable bags, trays and containers, food packaging, household articles, electrical insulation
High density polyethylene (HDPE)	Toys, houseware, shampoo, milk bottles
Polyvinyl chloride (PVC)	Window frames, pipes, electrical insulation, floor covering, toys
Polyurethane (PUR)	Building insulation, mattresses, insulation for fridges and other appliances
Polyethylene terephthalate (PET)	Bottles for different soft drinks, juices, water etc. Textile fibers, electrical insulation
Polystyrene (PS)	Food packaging, building insulation
Others	Polycarbonate (PC), polyamide (PA), optical fibers (PBT)

Plastic particles of less than 5 mm in size are usually defined as MP. The size of the MP particles is often attributed to several size ranges that vary between studies (13). There is no official definition of the lower size of MP particles, however NP considered particles below 100 nm (13). MP can have different shapes, such as spheres, fibers and irregular fragments. The high variability in shape, size and polymer makes it hard to define a common standard for environmental microplastic particles (1).

As plastic pollution has become one of today's biggest environmental crises, MP has been reported in marine, freshwater, and terrestrial ecosystems, from the sea surface to sediments, from beaches to the deep sea, from lakes to rivers, from the tropics to the poles (14), and are now ubiquitously present in the environment (13). Environmental MP can be differentiated into two different categories: (a) primary MP, which is produced in the small size range for its purposes, such as cosmetic applications, abrasion substances for air blasting and cleaning, decoration, vectors for drugs, soil additives, or industrial nurdles that are melted into end products, and (b) secondary MP, that occur when larger plastics debris or products in use degrade into MP, including wear abrasions, such as on tires and shoes, cleaning or paint removal abrasion, and weathering, such as of agricultural foils or abandoned garbage (15). The emission of secondary microplastics is more difficult to regulate than primary MP, due to the way they

are produced and the comprehensive number of primary MP (16). The first discovery of plastic litter in the ocean was at the start of 1970 and was paid minimal attention (17). Today, it is estimated that 270 million tons of plastic are floating in the oceans, and eventually will break further down into MxP. NP could be produced from the derived from the degradation of microplastic, or directly from industrial sources (13, 16).

1.1.1 Chemical additives, pollutants and plastisphere

As above mentioned, plastic products consist of various chemical substances enhancing functional properties, tailored for their use (10). The additives are added to the polymers to improve both shelflife and performance. The most utilized additives are flame retardants, plasticizers, antioxidants, pigments and light and heat stabilizers, especially important for food packaging (10). As the additives are not usually bound to the polymer itself, they could potentially migrate from the polymer into the surrounding environment (10). Despite how functional the additives are in creating the final plastic product, their potential to contaminate environmental media is a huge challenge. The magnitude of the emission is hard to identify, considering many factors affect the fate of the plastic. Therefore recycling plays a crucial part to make sure that the emission of the substances of concern is being reduced (10). Also, surface modifications are commonly used to alter the plastics material properties to modulate reactivity, hydrophilicity and to increase binding ability (1). Carboxylation and amination are an example of such modifications, which is the adding of a carboxyl or an amine group to the surface (1).

Moreover, MP in the environment can adsorb and serve as transporters for different types of organic contaminants, such as persistent organic pollutants (POPs) and heavy metals. POPs are toxic compounds, produced for the purpose of disease control, agriculture or manufacturing (18). However, these same chemicals have a negative effect on human health and the environment. They can be transported by water and air and persist long in the environment. POPs are unsusceptible for any type of degradation and will accumulate in the food chain (17, 19). Due to their hydrophobicity, they can be adsorbed onto the surface of plastic litter. The contaminated plastic particles can be transported along with the ocean current and further disturb other ecosystems. More importantly, it can be ingested by other marine species and transported through the food chain (17). In addition to chemicals, microbes and other organisms have been found on plastic debris. The term "plastisphere" describes the microbial community associated with floating plastic debris. The new human-made ecosystem serves as a habitat for

different types of microorganisms in the aquatic environment, including invasive species, pathogens (e.g., *Vibro spp*. and *Aeromonas salmonicida*) and harmful algae, by creating a long-lasting surface that can facilitate their growth (20).

1.1.2 Separation and analysis of microplastic

Currently, there is a wide range of analytic methods to identify microplastic, both in concentration and size, including microscopy, spectroscopy (Fourier-transform infrared spectroscopy FTIR, Raman) and thermal analysis (pyrolysis-gas chromatography-mass spectrometry, py-GC/MS) (Figure 2). However, prior to the identification of the MP particles, the samples need to be processed, and MPs should be separated from the matrix. The most widely used techniques for the isolation of MP include density flotation, filtration, and/or various digestion protocols (21). The complexity of the sample preparation implies also that the throughput is limited, as sample preparation can take several days. This variety of methods may compromise data quality and make it difficult to compare results from different studies (22). There is a necessity to standardize and validate a fast, reliable and inexpensive method for evaluating and detecting MPs in biological samples, with the final aim to establish wide monitoring programs (5). It is hard to assess the risk of MP without validated methods and reference material (13). The study of MP often implies the extraction of plastic from different complex matrices, ranging from water, soil, sediment, blood and feces from several species. One needs to eliminate the matrix prior to instrumental analysis, such as before FTIR, Raman or py-GC/MS (13).

Filtration is often a suitable method for the isolation of free MP or in relatively simple matrices, such as water. It is an inexpensive method and enables to categorize the particle size. However, its limitation is the possible loss of particles with large pore size filters and clogging of smaller filters. Different types of matrix digestion, such as alkaline, acidic and enzymatic digestion, are more suitable for more complex matrices (13). However, both alkaline and acidic digestion can damage the plastic particles and may need several optimization steps to digest all the matter. Enzymatic digestion yields less damage to the polymers, but is more expensive, variable enzymatic activity depends on the matrix and may also need careful optimization (13). These are all methods for sample preparation and are often the first step prior to different methods of detection, such as FTIR, Raman, py-GC/MS and fluorescent tagging. FTIR are a spectroscopic method that are based on how infrared (IR) radiation interacts with solid, gaseous or liquid samples. The FTIR measures both the frequency and intensity the sample will absorb. This

yields each sample its own "fingerprint" or absorption spectrum. This fingerprint can be used to compare and identify several MP polymers, by comparing their spectra with libraries (13, 23). The method is relatively easy, reproducible and do not require much sample preparation or number of samples. Also, the FTIR can identify the number of particles with sizes down to 2 μm, depending on the sensibility of the instrument (13). However, MP in the environment may have been subjected to UV-degradation or weathering. This may challenge the identification process, as environmental MP can differ from pristine plastic particles, and may deviate from the reference library. Also, these spectroscopic methods are expensive (13). Thermal analysis, such as py-GC/MS, is normally used in combination with mass spectroscopy and is an analytic tool that can identify a wide variety of polymers and materials (24, 25). Py-GC/MS is based on the heating of a sample that will be broken down into smaller fragments. The fragments are separated chromatographically, analyzed and then data is interpreted by skilled personnel. In this way, one is able to identify isolated plastic particles by assessing their thermal degradation (24, 25). The rapid heating process gives reproducible results. However, pyrolysis is very time-consuming and does not give any data on the particle size, shape or number (13).

Another important issue to consider when analyzing MP is to prevent contamination of the samples. Due to the ubiquitous presence, equipment like gloves and clothes but also the air may contain MP (13).
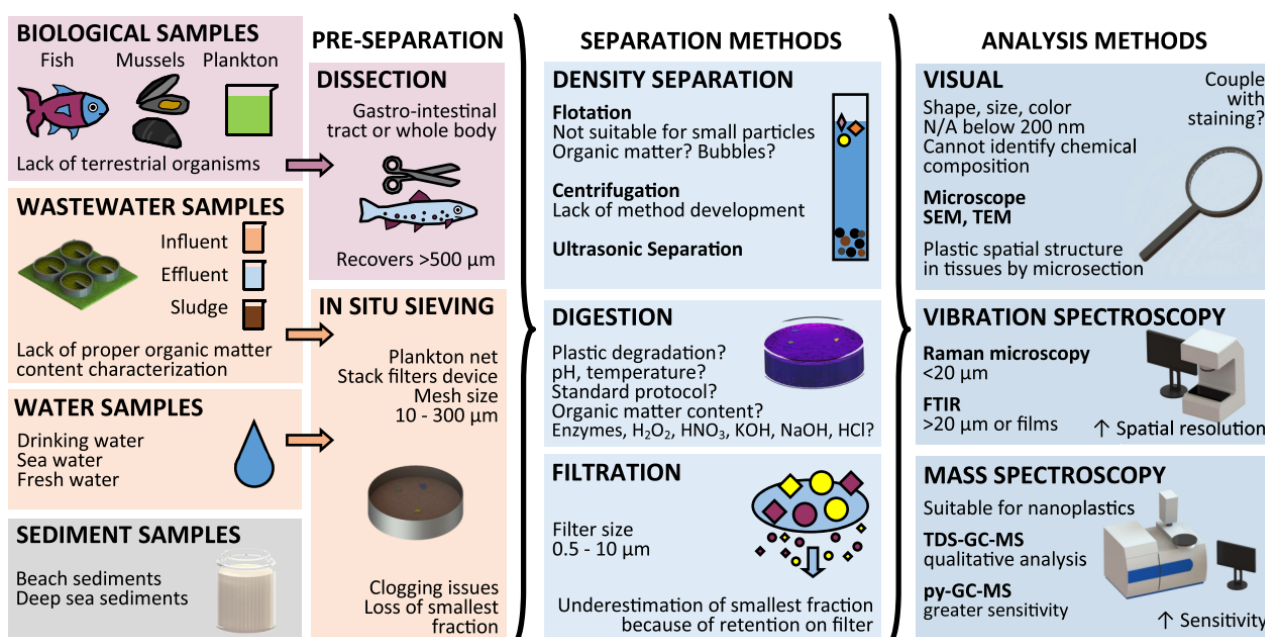
Figure 2. Overview of different types of separation and analysis methods for MP and NP. Figure is taken from a review by Nguyen and coauthors (26).

1.1.3 Health effects of microplastic

Most research on the health effects of MP is conducted on aquatic organisms, while knowledge of the potential risk and exposure of terrestrial animals and humans is less often investigated (27). Still, the existing literature on terrestrial organisms is mostly conducted in rodents. Indeed, in the last decade the focus on MP effects on terrestrial mammals, such as mice or rats has increased (9, 27, 28). Rodents can serve as model animals as their anatomy and physiology share similarities with humans. Therefore, the study of the biodistribution and bioaccumulation of MP in rodents is very useful and may elucidate its potential effect on humans (27). The available research on animals provides preliminary insight on MP's potential effects and the consequences of exposure. Most research on MP uptake and distribution in rodents is administered through food and drinking water, as it is the most likely route of exposure (28). When health effects of MP are discussed, it is still a matter of debate which MP can be absorbed, or whether the effects of MP are related to gastrointestinal disturbances. Indeed, absorption and translocation to other organs have been reported for particles of sizes up to 20 μm, while larger particles are usually not found in other organs than the gut (27). Data suggest that absorption and distribution of MP are depended on particle size (1, 27, 29). While fragments in the centimeter and millimeter range are a threat to the environment and are often considered too coarse to be absorbed, smaller MP and NP are a greater concern for most biological systems, as size limits both absorption and translocation (1, 27).

Several health effects have been investigated in rodents, such as inflammation, oxidative stress, and metabolic disruption (28). In turn, these effects may increase the risk of degenerative disorders such as cardiovascular disorder, autoimmune disease, cancer and gastrointestinal disorders (6). Also, other evidence has shed light on MP and NP and their ability to dysregulate signaling pathways and modify gut microbiota (6). In a study by Li and coauthors mice were fed PE (10-15 μm) with different concentrations ranging from 6, 60 and 600 μg per day (9). They reported an increased number of gut microbe species and bacterial abundance among the mice who were fed with MP compared to the control group, thus showing altered gut microbiota due to the high consumption of microplastic (9). Deng et al. studied the accumulation and distribution of two sizes (5 μm and 20 μm) of fluorescent PS in the gut, liver and kidneys of mice (27). PS was found in all investigated tissues. However, the 5 μm particles exhibited higher accumulation in both kidneys and gut, compared to the 20 μm particles (27). Another rodent study showed an accumulation of PS particles (50 nm – 3 μm) in the lungs and spleen

with an inverse correlation with uptake and size (29). Data from another study of mice fed with different sizes of PS (1, 4 and 10 μm) demonstrated minor uptake of PS particles, but the 1 μm showed higher cytotoxicity compared to the 4 μm and 10 μm particles (30).

1.1.4 Potential impact on human health

1.1.4.1 Exposure routes

MP can enter the human body through three different routes: ingestion, inhalation and dermal contact (31). Current research suggests that MP contaminates a large variety of food groups and beverages. Drinking water may also be a source of contamination due to direct contact with primary or secondary MP. Single-use plastic bottles may therefore contain as many as 15 MP particles in every liter (1). Indeed, MP has been found in tap and bottled water, beer, milk, tea, honey, salt, sugar, seafood, fruit and vegetables, indicating that it is ubiquitous in the food chain (5, 32). Human ingestion of MP through contaminated food has been estimated to be 39 000-52 000 particles per year (28). Indirect ingestion, also referred to as trophic transfer, may occur through the ingestion of organisms that contain MP, such as mollusks, crustaceans and fish fillet (1), which are known to contain MP and are also typically part of the human's diet. A recent review identified 26 studies that evaluated MP concentration in food and beverages consumed by American citizens. They found drinking water, seafood, sugars and inhalation to constitute a source to MPs (32).

The second route for human exposure is through inhalation. Airborne MP can enter the body through inhalation and deposit in the deep lung, where the gas exchange in the alveoli occurs. The size, shape and density of the MP would determine how and if the respiratory is affected (1, 31). Exposure studies have not yet been conducted, but airborne MP may cause respiratory diseases, such as bronchial reactions, asthma, allergies, diffuse fibrosis and inflammatory and fibrotic changes in the bronchial tissues. The toxicity may come from the plastic particles themselves or their leachates (33).

The last exposure route is through dermal contact (31). MP has been regularly used in several cosmetic products, such as sunscreen, hand lotion, skincare products and toothpaste prior to 2019 when new restrictions had been introduced by the Food and Drug Administration (FDA) to minimize MP discharge into the water system (1). However, the penetration of the stratum corneum, the outer layer of the epidermis, is limited to particles less than 100 nm. Therefore, due to the size of MP, it is unlikely that the absorption through the skin would occur. Nevertheless, NP could be small enough to potentially cross the dermal barrier (34). Currently,

there is not enough evidence to conclude whether there is a risk of absorption of plastic particles through this dermal exposure route (1).

1.1.4.2 Fate of microplastic in the human body

The fate of MP particles after ingestion is mostly unknown. There are no data demonstrating whether MP can translocate from the gut cavity or if MP particles are entirely excreted with the feces. Before reaching the intestinal epithelium, MP has to pass through several sections of the gastrointestinal tract that may change their physical and chemical properties and surface reactivity. There is no enzymatic degradation of MP (5). For absorption to occur, MP has to cross the intestinal epithelium in the intestine which mainly consists of enterocytes, goblet cells and M-cells (28). The fate of the microplastic following ingestion proposes different scenarios: (i) MP stays in the lumen and is excreted, (ii) MP crosses the intestinal epithelium paracellularly, (iii) MP is taken up by enterocytes, (iv) MP are taken up by other cell types such as M-cells or (v) MP stays inside the intestinal cells and do not enter the bloodstream (5). Another suggested mechanism for the uptake of MP may be through Peyer´s patches in the small intestine. Specialized cells in the Peyer`s patches could facilitate the transport of MP from the lumen towards the follicles via phagocytosis and ultimately reach the circulatory system (1, 35). There is great uncertainty about the absorption rates of ingested MP, which is probably dependent on the size, shape and solubility of the particles (32, 35). The European Food Safety Authority (EFSA) considers MP to have very low bioavailability, probably less than 0.3% (5). Therefore, a minimal part of the ingested or inhaled MP may be taken up and enter the bloodstream (36, 37). There is also little knowledge on the fate of the MP after entering the bloodstream, on the elimination routes, renal filtration or biliary excretion, or if they are deposited in other organs (37).

It has been shown that environmental MP such as PS, can also be modified through interacting with proteins. This modification creates a PS-protein complex due to Van der Waals interactions. The protein-coated complex is called a "corona" and may enable the MP to escape the immune system and persist in the circulation (1). The interaction of MP and NP with biological systems and their potential risk is not well understood (38). However, the protein corona has been identified with the binding of proteins involved in blood coagulation, lipid metabolism and complement systems (38).

1.1.4.2.1 Microplastic in feces

Precise information on the MP distribution in the human body is rare, however if MP is ingested through the diet feces may serve as an ideal non-invasive matrix for measuring MP contamination. It will also provide direct evidence of MP exposure by ingestion and proof of MP presence in the food chain (39). Indeed, the occurrence of MP in humans, wild animals, livestock and pet feces has already been documented in pioneering studies (39-43). However, there is no standardized method for extracting MP from human feces. One of the challenges for the extraction of MP in human feces is to directly distinguish the MP from organic and inorganic matrices (39). The extraction can be performed with digestion methods, which may include several chemicals such as nitric acid (HNO_3), hydrogen peroxide (H_2O_2), potassium hydroxide (KOH), sodium hydroxide (NaOH) and enzymes (39). Powerful chemical reactions and high temperature may damage the plastic, meaning other relative gentle methods needs to be carried out to preserve the plastic particles. A study by Yan et al. (39) introduced a novel approach trying to meet the criteria for preserving several types of plastic polymers in the feces of humans, chickens and zebrafish. By using Fenton's reagents and nitric acid to digest the feces samples and ethyl alcohol to remove the residues on the MP surface and allow easier identification of the particles (39). By using this method 97.8% of the MP was recovered, and no damage to the MP particles was observed with Raman spectrometry (39). The digestion step is important for digesting all the non-plastic matter in the feces sample, such as proteins, fat, non-digestible fats, bacteria and other solids (43).

1.1.4.2.2 Microplastic presence and consequences in human blood

While exposure studies in humans cannot be done for obvious ethical reasons, a few *in vitro* studies have been performed to study the effect of NP on isolated human blood cells (38, 44). Following ingestion both, MP and NP may enter the bloodstream. As of today, there is only one study demonstrating this in humans (37). Leslie and coauthors demonstrated the presence of four known polymers in 17 of the 22 healthy volunteers included in the study. This pioneering study of MP distribution in humans is of great concern and has demonstrated MP's ability to enter the human bloodstream (37). Blood is a suitable matrix due to its role as a transport pathway, and the ease of assessing samples directly from the body with minimal risk of contamination (37). After reaching the circulatory system, small MP may come in contact with circulating blood cells and platelets. Previous in vitro studies have investigated whether MP or NP affect hemolysis, platelet aggregation and immunoreactivity (38, 44-46). Some have

shown NP ability to induce platelet aggregation, thus presenting an increased risk for cardiovascular risk upon exposure (44, 45). In most of the studies, MP with surface modifications has been used.

Platelet aggregation is highly important for the coagulation system, which is either performed by coagulation factors (proteins) or by platelets. Platelet aggregation can be the cause of several diseases, among them thrombosis, heart attacks and strokes, depending on where the blood clot is formed (47). While platelet aggregation is a physiological process important for maintaining blood flow and continuous repair processes, thrombosis is an unintentional activation of the hemostasis (47). A thrombus can occur in both arteries and veins. When this happens in the arterial system, it is usually the platelets that cause the blood clot, while on the venous side, the coagulation system itself is of greatest importance (1, 47). An arterial thrombosis may lead to ischemic stroke and ischemic heart disease, while venous thrombosis may cause a pulmonary embolism. Venous blood clots mainly consist of fibrin and red blood cells, while arterial blood clots are composed of fibrin and platelets (1). Research has shown NP in blood to activate platelets, increasing aggregation and enhancing the formation of a thrombus (1, 48).

1.1.4.2.2.1 Platelet´s role and functions

Platelets are small cell fragments in the blood with high biological significance. They are produced from larger megakaryocytes and play a major role in of the hemostasis (46, 49). Platelets are important in response to a bleeding injury by forming a plug on the site of a vascular injury (50). They consist of lysosomes and several granules, which serve as deposits and secretory vesicles which release their content, such as inflammatory and vasoactive substances, upon vascular injury and platelet activation (46). During damage in the vascular wall, platelets adhere to the injured area and release their compounds when activated in order to facilitate normal physiological responses, such as wound healing, inflammation and hemostasis (49). Platelets normally interact with several white blood cells and form leukocytes-platelets aggregates (LPA), especially with neutrophils and monocytes. The binding of platelets to leukocytes occurs through platelet surface expression of P-selectin. This transmembrane protein facilitates the adhesion of activated platelets to neutrophiles and monocytes (50, 51). P- selectin is found in secretory granules in endothelial cells and megakaryocytes. When platelets are activated by the coagulation factor thrombin, P-selectin is translocated to the surface of the platelets plasma membrane (51).

Low levels of LPA are normally present in healthy individuals. However, the formation of LPA is increased among patients with cardiovascular diseases, such as myocardial dysfunction, coronary artery disease and chronic venous insufficiency (50). Also, elevated levels (>10%) of monocyte-platelet aggregates (MPA) have been found in patients with coronary heart disease (46). Hence, both LPA and MPA may serve as an *in vivo* marker for early platelet aggregation (46, 51). The function of platelets in whole blood can be assessed through flow cytometry and measure both platelet activation, aggregation and adhesion (51). McGuinnes and coauthors investigated PS latex nanoparticles (50 nm) with different surface derivation state and their potential to cause platelet aggregation in vitro with flow cytometry (44). They found all three groups, including aminated, carboxylated and unmodified PS, to induce the formation of MPAs. However, no differences between the groups were observed.

1.1.4.2.2.2 Flow cytometry

Flow cytometry (FC) is a precise and commonly used method in immunology, molecular biology, cancer biology and cell sorting (22). FC is used to count cells or particles in a liquid solution and is based on every cell or particle's unique fluorescence and their refractive properties (52). Liquid samples pass through light beams from one or more lasers and sensitive photomultiplier tubes measure both light scattering and fluorescence intensity from the particles, depending on their size, shape and autofluorescence (22). The detectors are used to assess the degree to which the cell bends by the light, forward scatter (FSC), and scatter the light, side scatter (SSC) (52). In this way, flow cytometry allows individual measurement, where SSC says something about the complexity/granularity and FSC about the size of the cells, and therefore provides detailed information on the characteristics of a cell or particle (22). In addition to examine the refractive properties of a cell, FC also allows us to measure the cells' expression of different types of antigens using monoclonal antibodies conjugated to fluorochromes (52). Fluorochromes are coloring chemical compounds that emit fluorescens after they have been illuminated, such as FITC, APC, PE etc. When the fluorochrome is irradiated with laser light, it is excited and emits fluorescent light in a specific spectrum. Detection of fluorescence in this spectrum is an indirect measurement of the degree of antibody-binding to the cell, and therefore gives us information about which antigens the cell expresses (52). Figure 3 gives an example of cell sorting of a human blood sample based on side scatter and forward scatter.
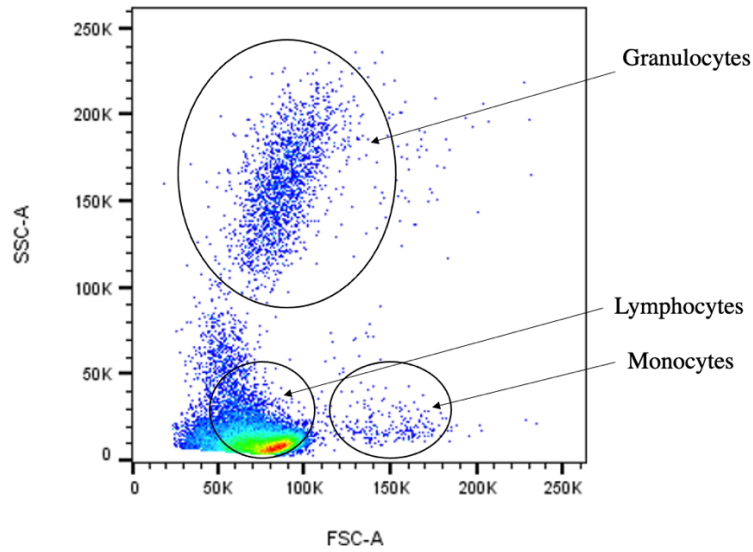
Figure 3. Different cell populations in human whole blood in flow cytometry. A human blood sample analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). The x-axis shows the forward scatter (FSC) and y-axis show side scatter (SSC). The FSC provides information about the cells size, while SSC describes the complexity/granularity of the cells. Granulocytes, monocytes and lymphocytes cell populations in human whole blood identified based on SSC-A and FSC-A. The figure was created in FlowJo (version10.8 BD Bioscience).

In whole blood flow cytometry different cell types can be distinguished by side scatter and forward scatter based on their size and complexity, as shown in Figure 3. However, the identification of different cell populations can be improved by using cell-specific antibodies (50). The presence of leukocytes associated with platelets can be detected with antibody staining for platelet-specific markers, such as activation-depended monoclonal antibodies. Each antibody is usually titrated in the laboratory for finding the optimal concentration (50, 51). The antibody targets a specific antigen on the cell of interest, and by flow cytometry technique we can determine the amount of antibody bound per cell. In flow cytometry analysis, monoclonal antibodies are preferable to polyclonal antibodies, as they will only bind to a single specific site on the antigen and provide more specific binding (51). The characteristics of platelets and other cells are therefore identified through light scattering and the fluorescence of the fluorophore conjugated antibodies (46). Analysis of circulating LPA involves the gating with a leukocyte-specific reagent, such as CD14-PE which is usually used for identifying monocytes and CD16- APC for identifying neutrophiles (50). A second antibody is then used to identify platelets, typically CD61, CD41 or CD42a. These second antibodies will distinguish platelet-

positive leukocytes from platelet-negative leukocyte subpopulations, due to the expression of antibodies on the surface of the platelets (50). Some antibodies are also targeted against P-selectin, such as CD62P. P-selectin is a protein that in resting platelet is sequestered within granules. However, when platelets are activated, they undergo physical modification and P-selectin is translocated on the surface of platelets, which can be then targeted by specific antibodies (46). Flow cytometry analysis have demonstrated that the expression of P-selectin correlates with the formation of LPA (46, 50). Previously, platelet surface expression of P-selectin has been considered the gold standard as an early marker of platelet aggregation (53). However, activated platelets will quickly lose the P-selectin attached to the surface. It has been demonstrated that LPA will persist detectable in blood for a significantly longer amount of time, and therefore are a more sensitive marker of platelet activation than P-selectin positive platelets (53, 54). LPA in whole blood measured by flow cytometry is usually identified as the percentage of total leukocytes that is both positive for a platelet-specific and a leukocyte-specific antibody (55). Thus, the use of flow cytometry could be a powerful analytic method to detect the potential thrombotic potential of MP in blood. Even if MP has only been described in a single study in blood, it is expected due to the increasing plastic pollution, that more such studies will appear. Activation of platelets and formation of LPA and MPA could be one potential mechanism of MP toxicity.

2   Hypothesis and aim of the study

The thesis had two overall aims:

First, it was to aim to investigate the occurrence of microplastic in human feces and relate the abundance of MP to dietary factors. Therefore, we wanted to investigate whether MP can be found in fecal samples from volunteers/patients following different diets, either rich in seafood or rich in meat.

Second, it was the aim to investigate potential effects of different MP polymers on platelet aggregates in whole blood in an ex vivo condition. Therefore, we investigated whether addition of MP to human blood from healthy volunteers caused platelet aggregation, both with neutrophils and monocytes.

The overall hypothesis of this thesis was that humans are consuming MP with their food or drinks, and MP can therefore be found in human gut and are present in human feces. MP can be demonstrated in human feces by FTIR, or pyrolysis-gas chromatography mass spectroscopy (py-CG/MS), and their amount is associated with the type of diet. Even though absorption routes are unclear at present, MP may occur in human blood and may act there as foreign bodies and can induce platelet activation and aggregation. This can be demonstrated in vitro in flow cytometry and the degree of platelet activation and aggregation is dependent on the concentration and the polymer type.

3 Materials and methods

3.1 Ethics

Feces were obtained from the CarbFunc study with ethical approval (clinicaltrials.gov identifier NCT03401970). An experimental investigation of platelet aggregation was done with blood samples obtained from the investigator who gave informed consent to blood drawing. Blood drawing was performed by trained personnel.

3.2 Study 1: Microplastic in human feces

3.2.1 Study participants and dietary assessment

Feces samples were collected from participants from the CarbFunc study, a dietary intervention study in obese participants within an age range of 20 to 55 years. Baseline data have been published by Horn et al. (56). Only baseline data were used in this study. Prior to the fecal sampling, participants reported their dietary intake by food records. From the dietary records, dietary pattern was obtained, and grouped according to the main food groups. The four groups were respectively meat, rice, pasta, pulses and oil (1); vegetables, fruit and seafood (2); sugary foods (3); and bread, cereal products and convenience food (4) (56). Based on our hypothesis on the risk of MP contamination we chose group 1 and group 2, hereafter named meat- and seafood-consumers respectively. Out of 192 participants at baseline, we selected participants with either high meat or high seafood intake. Ten samples of each group were chosen for the feces analysis. Table 2 gives an overview of the consumption groups and the amount of fecal material analyzed.

3.2.2 Feces collection

Originally the collection of the feces did not aim to analyze microplastic content, therefore patient did not follow any contamination prevention sampling method and every participant was handed out a plastic container for the feces collection, made up of PS. However, in the present study the type of polymer in the container would be taken into account in the results as a possible source of contamination.

3.2.3 Microplastic isolation from fecal samples

All equipment and chemicals used in this matrix digestion protocol are listed in the Appendix 3: Materials and suppliers.

Day 1:

Twenty fecal samples were weighed in glass containers. Two control samples were prepared without fecal material in order to check for any contamination during the procedure. The control samples were subjected to the same protocol as the fecal samples. Sartorius electronic semi-microbalance (R 180 D, Germany) 6-digit scale was used for weighing the fecal samples. Every metallic tool was washed carefully between each sample to avoid possible cross-contamination. 30 ml of the mixture of H_2O_2 15% and HNO_3 5% was added to the samples. Samples were covered with aluminum foil and incubated for 20 - 24 hours at 37°C and 225 rpm in a shaking incubator (Edmund Bühler GmBH, Germany).

Day 2:

The samples were filtered through PTFE filters (Mitex™ Membrane Filter, 5μm pore size, Merck) using a vacuum pump. All samples were washed with pre-filtered 0.01% (m/m) Tween®-20, ethanol-water (1:1, v/v) and then water. After filtration, the filter was placed inside the same glass container used previously. 30 ml of KOH 10% (m/m) was added to each container. Samples were covered with aluminum foil and incubated for 20 – 24 hours at 37°C and 225 rpm. After about 30 minutes, the filters were washed with KOH and placed in a glass container to be used again to filter the samples the following day.

Day 3:

Samples were filtered through the same PTFE filter used in the first step. The glass container and the samples were washed with 0.01% (m/m) Tween®-20, ethanol-water (1:1, v/v) and water. Then the filters were stored in Petri dishes until further analyses.

Table 2. Overview of the fecal samples analyzed for MP contamination. The average feces sample weighed 0.4453 g.

Group	Sample ID	Mass (gram)
Seafood	C105	0.4141
	C144	0.4753
	C125	0.4394
	C316	0.4048
	C306	0.4740
	C334	0.3864
	C065	0.3347
	C081	0.4731
	C080	0.3680
	C049	0.4096
Meat	C014	0.4500
	C025	0.5754
	C094	0.4906
	C127	0.5348
	C341	0.5344
	C090	0.4195
	C013	0.4334
	C098	0.4258
	C111	0.4425
	C052	0.4195
Control	Ct.1	-
	Ct.2	-

NORCE, Stavanger

The PTFE filters were brought to the microplastic lab in NORCE in Stavanger to be analyzed with FTIR. The PTFE filters were placed in a glass beaker with 50 ml of pre-filtered EtOH: H2O (1:1, v/v) mixture and sonicated for 10 minutes to suspend all the particles trapped on the filters. After sonication, the filters were washed with $EtOH:H_20$ and left aside. The suspension was then evaporated to a final volume of circa 2 ml. The concentrated solution was finally filtered through Whatman Anodisc inorganic filter membrane (pore size 0.02 μm, Merck) and left to dry in a glass petri dish. Figure 4 provides an overview of the sampling process.

3.2.4 Microplastic identification and quantification

It was planned that MP was characterized by FTIR and py-GS/MS analyses at the MP laboratory at NORCE in Stavanger. These two techniques are often used in combination when there are complex environmental matrices, such as feces which consist of a lot of organic matrix compounds (24). Finally, samples were only analyzed by FTIR due to technical problems.

3.2.4.1 Fourier transform infrared spectroscopy

MP was characterized by FTIR analysis at NORCE´s microplastic laboratory in Stavanger. However, the protocol for the FTIR is taken from a paper by Kirstein and coauthors, a study of quantification and quantification of MP in drinking water (57). NORCE provided this paper, but not the exact protocol. As we did not receive the FTIR protocol from NORCE for these analyses, some of the settings from the machine may differ from what was actually performed. The quantification of the MP particles from the fecal samples was determined using a Focal Plane Array (FPA) based on FTIR technique. Using a Cary 670 IR spectroscope coupled with a Cary 620 FTIR microscope, all areas of the filters were scanned. The microscope, with its 25x Cassegrain objective, produced a 3.3 μm pixel resolution on a 128x128 mercury cadmium telluride (MCT) FPA detector. Transmission mode was used for all scans with a spectral range of 3750-850 cm^{-1}, and a resolution of 8 cm^{-1} (57).

3.2.5 Quality assurance and quality control (QA/QC)

Given the ubiquitous presence of MP there is a risk of contamination in the laboratory. Due to this, a cotton lab coat and nitrile gloves were always used during sample processing, digestion and analysis. Plastic free equipment such as glass containers, metallic spoons, spatula and tweezers was used in every step and carefully cleaned with Milli-Q water between each sample. The equipment in contact with the samples or the chemical solutions was washed before use

and then covered with aluminum foil to avoid microplastic airborne contamination. All the chemicals used in the protocol were pre-filtered with Whatman™ GF/C (1.2 μm pore size) and kept in glass containers with glass lids. Also, to minimize the risk of contamination the samples in glass containers were covered with aluminum foil during all the procedures. All the steps in the protocol were performed in a ventilated closed cabinet, which was wiped with ethanol and paper prior to the practical work. Positive and blank control were carried out in triplicate. The positive feces samples were spiked with a known amount of microplastic, and recovery rates were analyzed. Blank controls, without feces, were carried out in duplicate purpose of evaluating the procedure and background contamination.
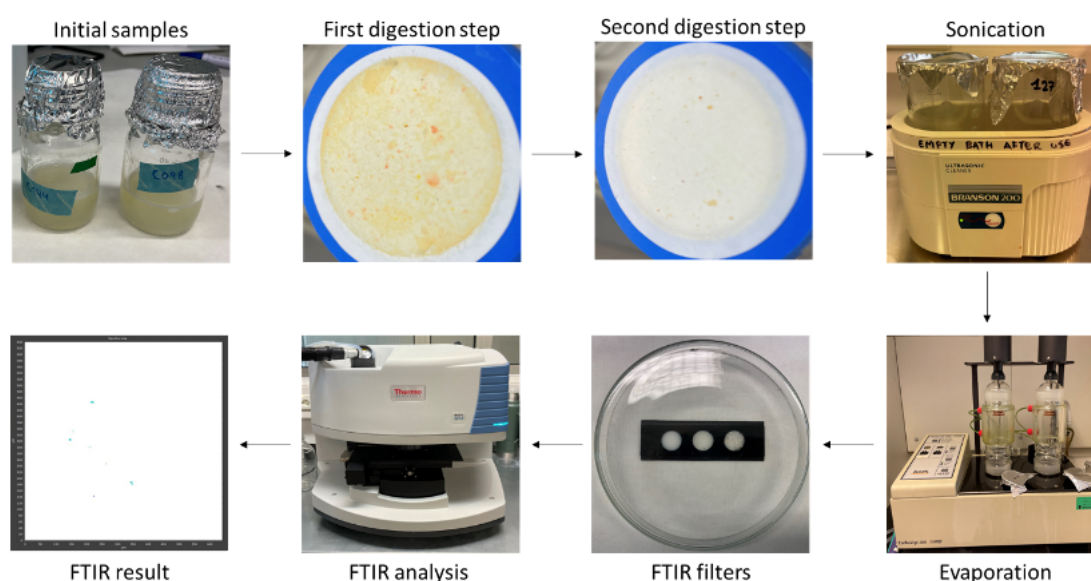

Figure 4. Graphic overview of the feces samples processing. From the initial preparation step with two digestion steps, followed by the evaporation of the samples and the FTIR analysis.

3.4 Study 2: Microplastic and cell aggregation in human blood

3.4.1 Blood collection

Blood was collected from four healthy volunteers with no reported medication for at least a week before the blood collection. The blood was collected into sodium citrate vacutainer tubes (BD Biosciences) to avoid any clotting in the samples and mixed by repeated gentle inversions.

3.4.2 Microplastic used in the study

Four plastic polymers were tested for blood toxicity experiment: PA, PS, PVC and PMMA. Spherical PA particles (Goodfellow Cambridge Ltd.) with an original size range of 5-50 μm were filtered in order to isolate the fraction <25 μm. PS, PVC and PMMA particles were produced at the Institute of Marine Research, Bergen. Briefly, plastic beads (2-3 mm size) were processed with a cryo-mill and planetary ball mill in order to obtain particles with a size range of <200 μm. The MP were then size fractionated through wet sieving to isolate the size fraction of interest (<25 μm).

3.4.3 Flow cytometry analysis and optimization steps

All samples were analyzed with a LSR Fortessa™ Cell Analyzer Flow cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). The cytometer was calibrated every day with CST calibration beads (BD Bioscience). Before samples were acquired, a fully stained sample was check on the machine to set the forward scatter and side scatter parameters and check if the signal was on scale. Compensation beads (ThermoFiscer scientific) stained with the respective antibody were used as single stained controls to calculate the compensation matrix. Compensation is necessary for correcting spectral overlap when using several fluorochromes. Compensation beads are often used instead of cells because it is reliable, easy to use and you will have more samples for your analysis, especially when you don't have big samples sizes cells to work with. Samples were run on low flow rate and up to 100.000 events were recorded. Gating strategy for platelet-neutrophile aggregates (PNA) are shown in Figure 6 and Figure 7, and for MPAs is shown in Figure 8 and Figure 9. Data were then further analyzed with software FlowJo v10.8 (BD Bioscience).

3.4.4 Labelling of blood samples

3.4.4.1 Antibody titration

Antibodies used in this experiment were CD16-APC, CD14-PE and CD42a-FITC (Thermo Fischer Scientific). All the antibodies were titrated in order to find the optimal concentration. The manufacturer suggested using a concentration of 5 µl of antibody in a final volume of 100 µl. Usually, this concentration is higher than what is needed, resulting in a higher cost of the material. For CD16-APC and CD14-PE 4 different concentrations (5, 2.5, 1.25 and 0.625 µl in a final volume of 150 µl) were tested, while for CD42a, five concentrations (5, 2.5, 1.25, 0.625 and 0.375 µl in a final volume of 150 µl) were tested. An unstained sample was processed in the same way. Firstly, a stock solution with 10 µl of antibody and 90 µl of Hepes-Tyrode buffer were prepared. A serial dilution (1:2) were made and the volume were adjusted to 100 µl with the buffer. Then 50 µl of whole human blood were added to the solution and incubated it for 30 min in dark at room temperatures. Further, 500 µl of 1X BD FACS Lyse (BD Biosciences) to fix the cells and lyse the erythrocytes were added. Then, the solution was analyzed with flow cytometry and 50000 events were recorded. The program FlowJo was used to analyze the data and finally the optimal concentration for each antibody was selected as shown in Figure 5.
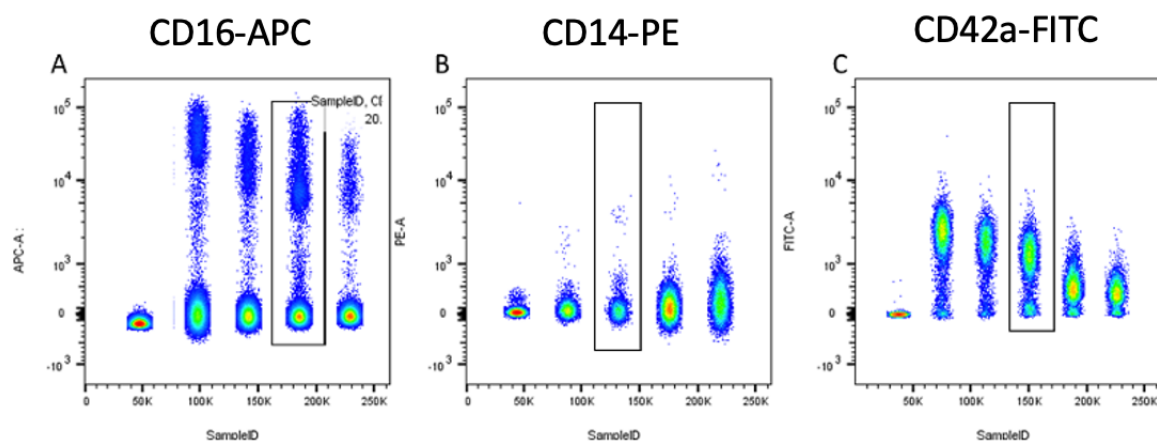
Figure 5. Titration of the three antibodies used in the experiment. Respectively CD16-APC (A), CD14- PE (B) and CD42a-FITC (C). Four, 5, 2.5, 1.25 and 0.625 µl, and five, 5, 2.5, 1.25, 0.625 and 0.375 µl, concentrations of the different antibodies, and one unstained, were analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). The black quadrant identifies the optimal concentration for each antibody, as for CD16 and CD14 were 2,5 µl, while for CD42a was 1,25 µl. Figure created in FlowJo (version10.8 BD Bioscience).

3.4.4.2 Staining method optimization

Three different antibody staining methods, retrieved from the literature (58, 59), were tested and the optimal one was then chosen for this study. The initial preparation of the samples was the same for the three methods, 50 μl of blood was mixed with the optimal concentration of antibodies resulted from the titration experiment and Hepes-Tyrode buffer was added to a final volume of 150 μl. The antibodies-blood solution was then incubated at room temperature (RT) in the dark for 30 min. Then, 500 μl of FACS lyse solution was added and mixed.

Method 1: after the addition of FACS lyse the solution was incubated at RT in dark for 15 minutes and centrifuged for 5 minutes at 280g. The supernatant was discarded, and the pellet was resuspended in Hepes-Tyrode buffer and finally analyzed through Flow cytometry (58).

Method 2: the solution was mixed and analyzed immediately with Flow cytometry (59).

Method 3: adapted from method 2. The solution was incubated again at RT in the dark for 15 minutes prior to Flow cytometry analyses.

3.4.5 Platelet-leukocytes aggregates assay

Five concentrations (approx. 500, 250, 125, 62.5 and 31.25 μg/ml) of plastic were serial diluted and tested in duplicate, and named C1, C2, C3, C4 and C5. Each concentration was tested for every polymer, respectively PMMA, PA, PVC and PS. Two controls (no MP) and two unstained (no antibodies) were also included in the analysis. The different concentrations of polymers were each mixed together with 50 μl Hepes-Tyrode buffer. An antibody cocktail consisting of 1.25 μl CD42-a FITC, 2.5 μl CD14-PE and 2.5 μl CD16-APC was added to 50 μl of whole blood. The plastic polymers were added to the whole blood and mixed gently. The solution of blood, antibodies, buffer and MP were then processed following method 3 (see staining method optimization section) as it gave the best results compared with the other two donors. This was done with 4 different donors.

3.4.5.1 Neutrophils-platelet aggregates

The neutrophiles were identified based on their SSC-A and FSC-A properties, as shown in Figure 6. A neutrophile-specific marker, CD16-APC, was used for selecting CD16-positive cells. Another marker for platelet-positive cells, CD42a-FITC, was used for selecting CD42a-positive cells. Data from flow cytometry analyses were examined with FlowJo v10.8 (BD Bioscience). Four populations were identified in separate quadrants: CD16 positive/CD42a

negative (Q1), CD16 positive/CD42a positive (Q2), CD16 negative/CD42a positive (Q3) and CD16 negative/CD42a negative (Q4). Quadrant 4 is both positive for neutrophile- and platelet-specific markers, and thus showing the PNA formed. The gating strategy is shown in Figure 7. This was done for all polymers in all concentrations.
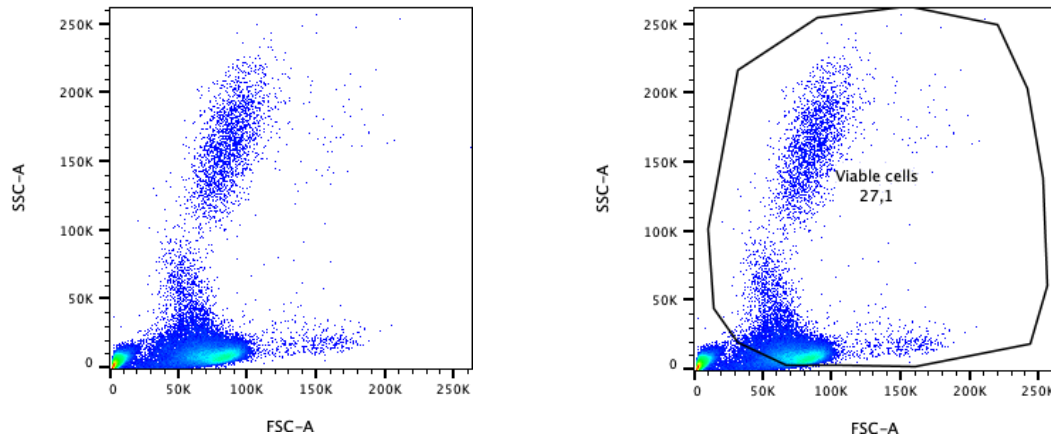
6a   **6b**

Figure 6: Leukocyte- and platelet population in human blood visualized by flow cytometry, FSC-A and SSC-A. Figure 6a shows a control blood sample with no added MP analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). Figure 6b shows gating of the viable cells and excluded debris, also by FSC and SSC. Neutrophils were stained with CD16-APC and platelets with CD24a-FITC. 100000 events were recorded. Figures created in FlowJo (version10.8 BD Bioscience).

Figure 7: Neutrophil-platelet aggregates in human blood. A quadrant plot with four different populations analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). X-axis is CD42a-FITC, and y-axis is CD16-APC. Q1 shows CD16-APC cells only, Q2 shows double positive cells, Q3 shows CD42a-FITC positive cells, and Q4 is double negative. PNA was identified as CD42a$^+$-CD16$^+$ and is clustered in quadrant 2 (Q2). The number under the quadrant name is the percentage of all gated cells. Figure created in FlowJo (version10.8 BD Bioscience).

3.4.5.2 Monocytes-platelet aggregates

The population of monocytes was identified based on their SSC-A and FSC-A properties, as shown in Figure 8. A monocyte-specific marker, CD14-PE, was used to confirm the CD14-positive cells in the gate. Another marker for platelet-positive cells, CD42a-FITC, was used for selecting CD42a-positive platelets. FlowJo v10.8 (BD Bioscience) was used to set the quadrant gates for the following populations: CD14 positive/CD42 negative (Q1), CD14 positive/CD42 positive (Q2), CD14 negative/CD42a positive (Q3) and CD14 negative/CD42a negative (Q4). Quadrant 1 is CD14 positive/CD42a negative and is gating only monocytes. Quadrant 2 is both positive for the monocyte- and the platelet-specific marker, and thus showing the % of MPA formed. Quadrant 3 is negative for CD14 and positive for CD42a, and gating platelets both single and aggregates (44). Quadrant 4 is negative for both CD14 and CD42a. The gating strategy is shown in Figure 9. This was done for all polymers in all concentrations.

8a   **8b**
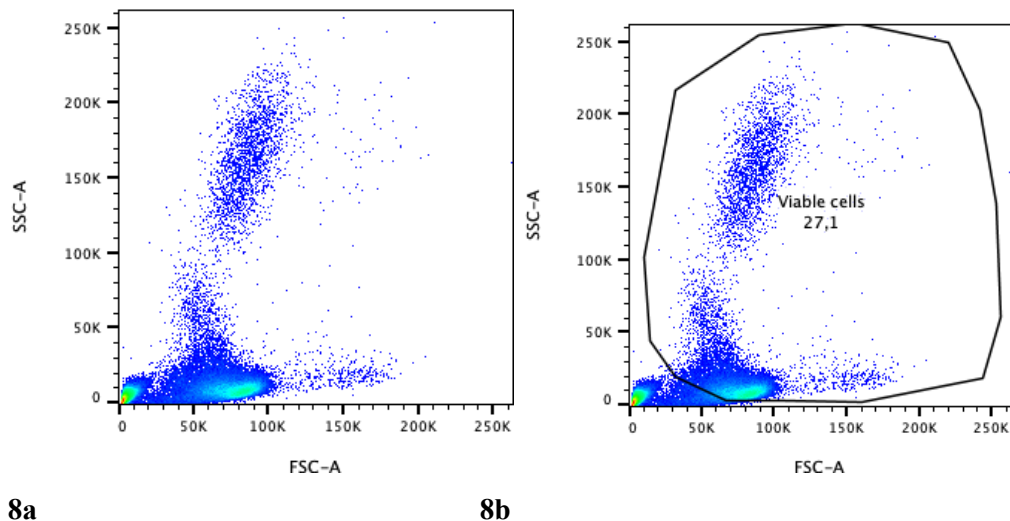
Figure 8. Leukocyte- and platelet population in human blood gated based on FSC and SSC. Figure 8a shows a control blood sample with no added MP analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). Monocytes were stained with CD14-PE and platelets with CD24a-FITC. 100000 events were recorded. Figure 8b shows the viable cells that were gated based on forward scatter area (FSC-A) and side scatter area (SSC-A). Figures created in FlowJo (version10.8 BD Bioscience).
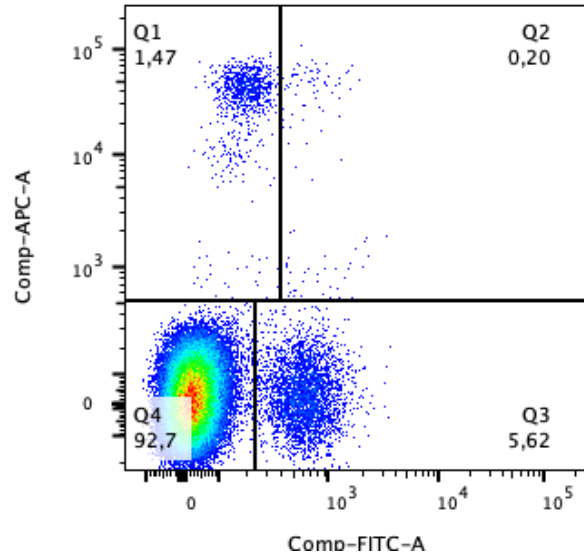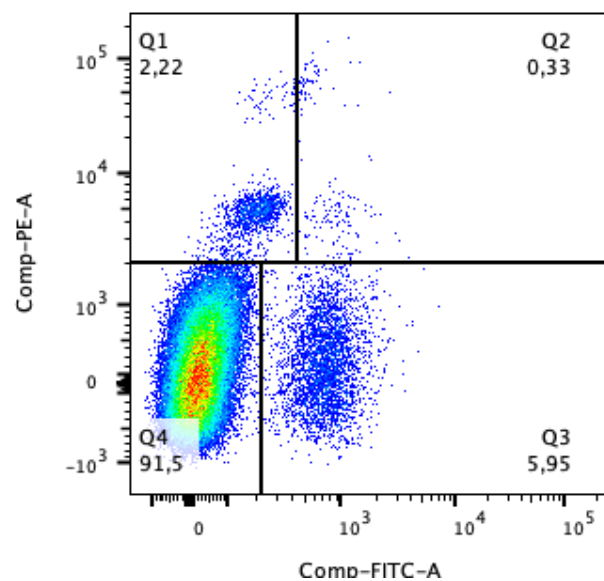

Figure 9. Monocyte-platelet aggregates in human blood. A quadrant plot with four different populations, analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). X-axis is CD42a-FITC, and y-axis is CD14-PE. Q1 shows CD14-PE cells only, Q2 shows double positive cells, Q3 shows CD42a-FITC positive cells, and Q4 is double negative. Monocyte-platelet aggregates was identified as CD42a+-CD14+ and is clustered in quadrant 2 (Q2). The number under the quadrant name is the percentage of all gated cells. Figure created in FlowJo (version10.8 BD Bioscience).

4   Results

4.1 Study 1: Microplastic in human feces

All fecal control samples were pretreated and then brought to NORCE microplastic laboratory in Stavanger for the last treatment step and FTIR and py-GC/MS analysis. Due to technical problems, only FTIR analyses were performed and presented in this work.

Table 3. Microplastic content in human feces analyzed with FTIR. Consumption group, type of polymer, size range and total mass

Sample ID	Group	MP	Number and polymer	Size range (μm)	Total mass (ng)
C105	Seafood	MPs	2 (PP, PE)	1.6 – 3.2	0.00794
C144	Seafood	MPs	1 (PE)	2.4 – 3.2	0.00555
C125	Seafood	MPs	21 (PP, PE, PS)	1.3 – 51.2	1.76275
C316	Seafood	Dirty	-	-	-
C306	Seafood	MPs	10 (PP, PE, PS)	1.1 – 51.2	1.50577
C334	Seafood	Dirty	-	-	-
C065	Seafood	Dirty	-	-	-
C081	Seafood	Dirty	-	-	-
C080	Seafood	Dirty			
C149	Seafood	Not reported			
C014	Meat	MPs	5 (PP, PE)	1.4 – 326.5	808.13
C025	Meat	Dirty	-	-	-
C094	Meat	MPs	1 (PP)	1.3 – 8.3	0.00239
C127	Meat	MPs	4 (PP)	1.3 – 4.2	0.01737
C341	Meat	MPs	2 (PP)	7.3 – 376	493.797
C090	Meat	Dirty	-	-	-
C013	Meat	Dirty	-	-	-
C098	Meat	Dirty	-	-	-
C111	Meat	MPs	2 (PE)	34 – 92.2	1.241
C052	Meat	MPs	11 (PP, PE, PS)	1.3 – 8.3	140.61
Ct.1	Control	No MPs	-	-	-
Ct.2	Control	No MPs	-	-	-

PP: polypropylene; PS: polystyrene; PE: polyethylene

A total of 20 fecal samples were digested and then analyzed using FTIR (Table 3). However, despite the optimization of the digested protocol, 9 of the samples could not be analyzed with the FTIR due to high background noise which did not allow any quantification of the particles. This was due to a layer of oxidized protein present on the filter. The personnel at NORCEs laboratory made several attempts to change the acquisition on the FTIR, but the IR did not manage to pass across the layer of oxidized proteins (Figure 11). Therefore, these samples will not be considered in the discussion of the results as it was impossible to conclude whether they contained MP or not. Also, 1 sample was not reported. No MP were detected in the quality control samples. All the 10 remaining samples which were suitable to be analyzed with the FTIR, contained microplastic. Of these 10 samples containing MP, the abundance of MP varied from 1 to 20 particles in each sample with size from 1.1 to 376 μm. No plastic particle larger than 376 μm was detected, and particles smaller than 1.1 μm were not found. The total mass of all the MP particles per participant ranged from 0.002 to 808 ng. The FTIR analysis demonstrated the presence of 3 different polymers, respectively PP, PE and PS. PP was detected in 8 out of 10 samples, PE in 7, while PS was present in 3 of the samples. In total, 59 particles of MP were found from the 10 feces samples. 24% of polymers were PP, 19% PS and 57% of them were PE (Figure 10). All three polymers were present in 3 of the samples, while two of them contained only 1 MP. When analyzed per diet, the fecal samples from the seafood (n=4) contained 1 to 21 particles of MP, with a size range of 1.1 to 51.2 μm and a mass of 0.005 to 1.763 ng, while the fecal samples from the meat group (n=6) contained 1 to 11 particles, with a size range of 1.3 tot 376 μm and a mass of 0.002 to 808 ng. Due to the limited number of data, no statistical analysis was performed.
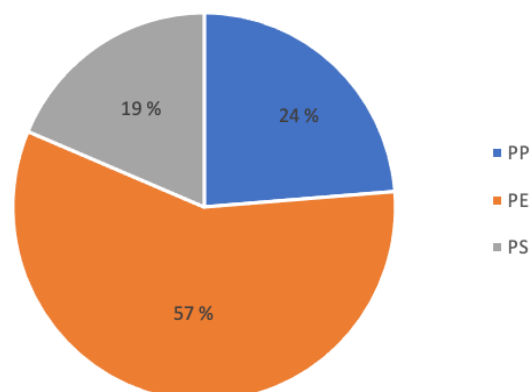
Figure 10. The percentage of the detected polymers in fecal samples from the participants (n=10) of the dietary intervention study.
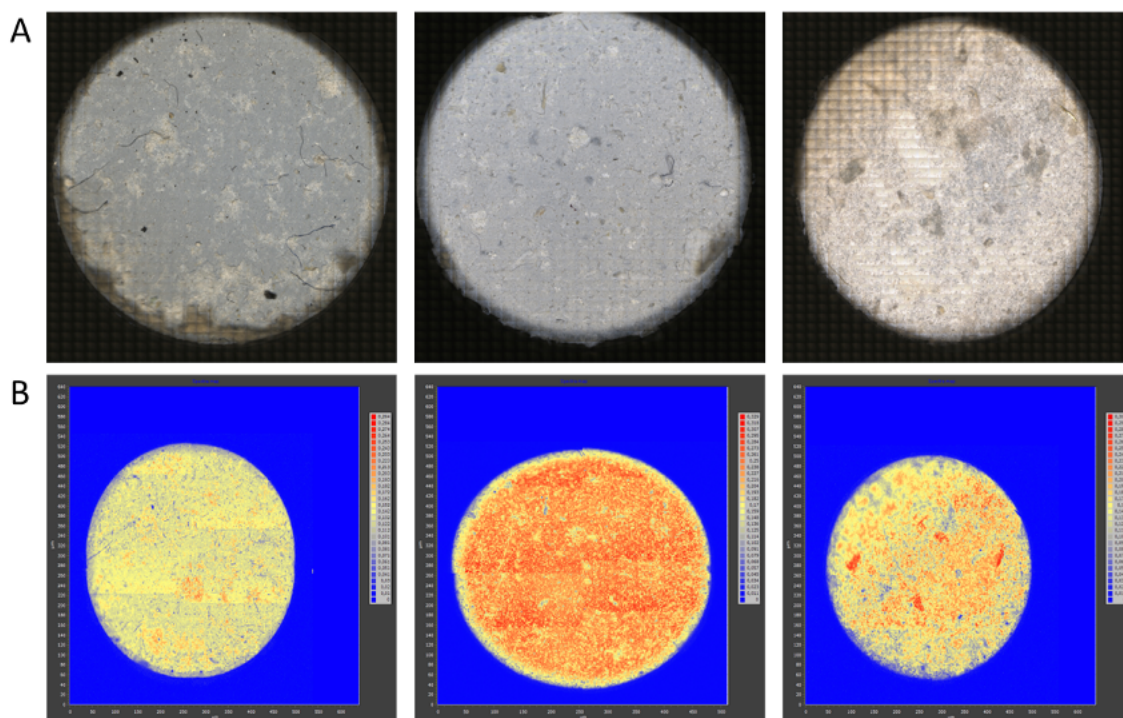
Figure 11. Example of human fecal samples with high background noise. Visual mosaic reconstructed image of a sample (A) and IR scan heat map (B). The layer of oxidized protein clearly visible in the heat map did not allow any further analysis with the FTIR.
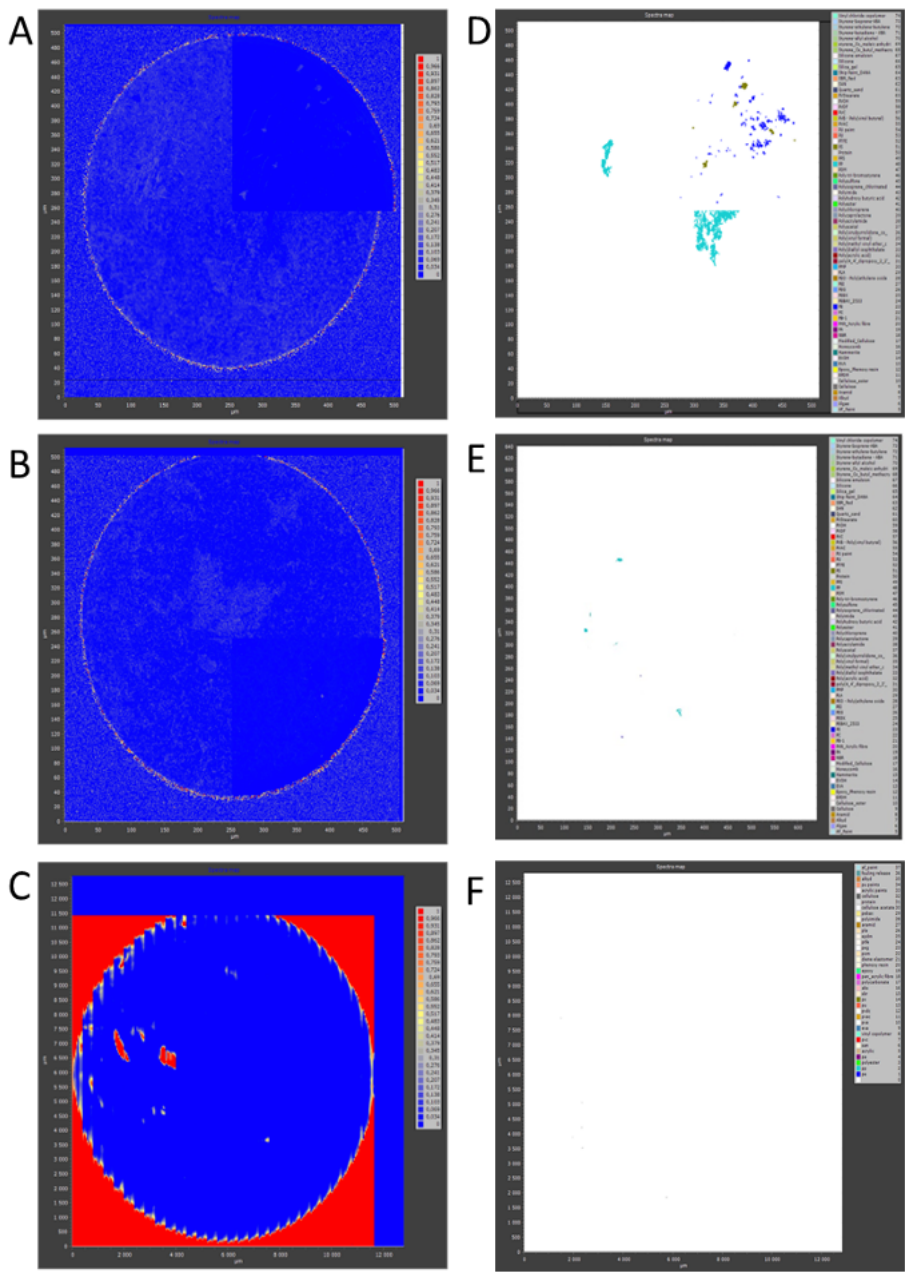
Figure 12. Example of human fecal sample analyzed with the FTIR. IR scan heat map of two fecal samples (A, B) and one quality control sample (C) and their respective SimPle map (D, E, F). Each polymer group is highlighted by a different color.

4.2 Study 2: Microplastic and cell aggregation in human blood

All polymers, respectively PA, PMMA, PS and PVC, were tested in duplicate in 5 different concentrations, approx. 500 (C1), 250 (C2), 125 (C3), 62.5 (C4) and 31.25 (C5) µg/ml, with particles in the 1-25 µm size range. Control and unstained samples were also measured. Table 4 and Table 5 shows the data exported from FlowJo and displays the % of MPA and PNA, as shown in Figure 13, presented as the mean and SD (standard deviation).

Table 4. The percentage platelet-neutrophile aggregates of all polymers and donors. The data are presented as the mean $\pm$ SD.

Polymer	Sample	Donor 1	Donor 2	Donor 3	Donor 4
PA	Control	0.23 ± 0.037	0.201 ± 0.079	0.398 ± 0.089	0.338 ± 0.033
	C1	0.33 ± 0.042	0.27 ± 0.042	0.345 ± 0.233	0.16 ± 0.071
	C2	0.335 ± 0.007	0.245 ± 0.035	0.275 ± 0.106	0.15 ± 0.042
	C3	0.275 ± 0.035	0.24 ± 0.014	0.57 ± 0.057	0.3 ± 0
	C4	0.11613 ± 0.161	0.22 ± 0.085	0.48 ± 0.212	0.102 ± 0.012
	C5	0.21 ± 0.014	0.155 ± 0.007	0.555 ± 0.106	0.125 ± 0.005
PMMA	Control	0.23 ± 0.037	0.201 ± 0.079	0.398 ± 0.088	0.338 ± 0.032
	C1	0.3 ± 0.099	0.14 ± 0.042	0.955 ± 0.068	0.16 ± 0
	C2	0.225 ± 0.021	0.19 ± 0.014	0.745 ± 0.360	0.17 ± 0.056
	C3	0.255 ± 0.049	0.185 ± 0.049	0.87 ± 0.184	0.305 ± 0.064
	C4	0.215 ± 0.021	0.19 ± 0	0.385 ± 0.134	0.305 ± 0.035
	C5	0.235 ± 0.021	0.2 ± 0.042	0.485 ± 0.134	0.275 ± 0.049
PS	Control	0.23 ± 0.037	0.201 ± 0.068	0.398 ± 0.088	0.338 ± 0.033
	C1	0.17 ± 0.014	0.195 ± 0.007	0.255 ± 0.035	0.16 ± 0.014
	C2	0.135 ± 0.007	0.33 ± 0.113	0.385 ± 0.148	0.24 ± 0.155
	C3	0.235 ± 0.035	0.119 ± 0.058	0.345 ± 0.134	0.245 ± 0.063
	C4	0.19 ± 0.028	0.18 ± 0.085	0.3 ± 0.042	0.2 ± 0
	C5	0.225 ± 0.007	0.145 ± 0.021	0.315 ± 0.007	0.235 ± 0.021
PVC	Control	0.23 ± 0.037	0.201 ± 0.079	0.398 ± 0.088	0.338 ± 0.032
	C1	0.205 ± 0.021	0.165 ± 0.035	0.42 ± 0.127	0.17 ± 0.042
	C2	0.215 ± 0.035	0.2 ± 0.042	0.735 ± 0.304	0.275 ± 0.191
	C3	0.225 ± 0.035	0.19 ± 0.070	0.355 ± 0.035	0.315 ± 0.063
	C4	0.235 ± 0.007	0.235 ± 0.035	0.29 ± 0.014	0.41 ± 0
	C5	0.195 ± 0.021	0.175 ± 0.007	0.605 ± 0.389	0.425 ± 0.007

PA: polyamide; PMMA: poly (methyl methacrylate); PS: polystyrene; PVC: polyvinyl chloride

C1= 500 µg/ml; C2= 250 µg/ml; C3= 125 µg/ml; C4= 62.5 µg/ml; C5= 31.25 µg/ml

Table 5. The percentage monocyte-platelet aggregates of all polymers and donors. Data are presented as the mean ± SD. Monocyte-platelet aggregates for polyamide in donor 4 was excluded, see Figure 3S in the Appendix.

Polymer	Sample	Donor 1	Donor 2	Donor 3	Donor 4
PA	Control	0.32 ± 0.06	0.368 ± 0.105	0.496 ± 0.041	-
	C1	0.4 ± 0.085	0.395 ± 0.007	0.4 ± 0.339	-
	C2	0.375 ± 0.007	0.49 ± 0.042	0.36 ± 0.028	-
	C3	0.32 ± 0.042	0.46 ± 0.14	0.57 ± 0.127	-
	C4	0.135 ± 0.191	0.28 ± 0.042	0.49 ± 0.141	-
	C5	0.225 ± 0.035	0.215 ± 0.007	0.635 ± 0.064	-
PMMA	Control	0.32 ± 0.06	0.368 ± 0.105	0.496 ± 0.041	0.101 ± 0.046
	C1	0.67 ± 0.551	0.43 ± 0.014	0.845 ± 0.813	0.225 ± 0.035
	C2	0.415 ± 0.021	0.465 ± 0.049	0.875 ± 0.134	0.165 ± 0.077
	C3	0.38 ± 0.056	0.405 ± 0.035	0.965 ± 0.205	0.19 ± 0.014
	C4	0.345 ± 0.078	0.425 ± 0.021	0.545 ± 0.148	0.12 ± 0.014
	C5	0.285 ± 0.021	0.4 ± 0.071	0.585 ± 0.021	0.21 ± 0.028
PS	Control	0.32 ± 0.06	0.368 ± 0.105	0.496 ± 0.041	0.101 ± 0.046
	C1	0.195 ± 0.021	0.33 ± 0.028	0.335 ± 0.007	0.225 ± 0.035
	C2	0.155 ± 0.007	0.705 ± 0.289	0.53 ± 0.18	0.165 ± 0.078
	C3	0.265 ± 0.078	0.325 ± 0.176	0.42 ± 0.113	0.19 ± 0.141
	C4	0.22 ± 0.071	0.455 ± 0.12	0.4 ± 0.014	0.12 ± 0.141
	C5	0.28 ± 0.014	0.38 ± 0.056	0.405 ± 0.021	0.21 ± 0.028
PVC	Control	0.32 ± 0.06	0.368 ± 0.105	0.496 ± 0.041	0.101 ± 0.046
	C1	0.195 ± 0.007	0.165 ± 0.077	0.435 ± 0.106	0.051 ± 0.008
	C2	0.215 ± 0.035	0.35 ± 0.056	0.875 ± 0.601	0.087 ± 0.046
	C3	0.25 ± 0.056	0.295 ± 0.148	0.525 ± 0.134	0.118 ± 0.044
	C4	0.265 ± 0.007	0.33 ± 0.028	0.36 ± 0.056	0.12 ± 0
	C5	0.195 ± 0.035	0.185 ± 0.091	0.285 ± 0.106	0.155 ± 0.035

PA: polyamide; PMMA: poly (methyl methacrylate); PS: polystyrene; PVC: polyvinyl chloride

C1= 500 µg/ml; C2= 250 µg/ml; C3= 125 µg/ml; C4= 62.5 µg/ml; C5= 31.25 µg/ml

The samples were marked as control, C1, C2, C3, C4 and C5. The control did not contain any MP and each control was used as baseline reference for every polymer with the same donor. A serial dilution was made, where the highest concentration of MP was marked as C1, with half of the previous concentration in C2, and further down to C5 with the lowest concentration of MP. The data in the column from sample 1, 2, 3, 4 and 5 is the percentage of the gated cells in quadrant 2 (Q2) as shown in Figure 13. All samples were tested in duplicate, therefore all the numbers in the cells in Table 4 and Table 5 are the mean of the duplicates. Also, the standard deviation was calculated to find out how much the samples differed from the mean.
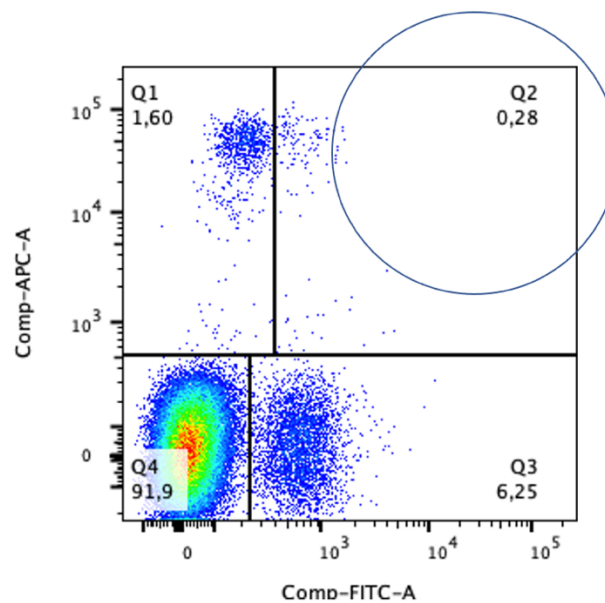
Figure 13. The percentage of the gated leukocyte-platelets aggregates, as shown for neutrophils. A control human blood sample with no added microplastic, analyzed with a LSR Fortessa™ Cell Analyzer Flow Cytometry (BD Biosciences) with a BD FACSDIVA™ v8.0 operating software (BD Bioscience). The circle is showing the quadrant that gates the platelet-neutrophile aggregates. The number in the quadrant is the percentage of all cells both positive for CD16-APC and CD42a-FITC. All percentage of neutrophiles- and monocyte-platelet aggregates are listed in Table 4 and Table 5. Figure created in FlowJo (version10.8 BD Bioscience).

Illustrated graphs of the percentage of the PNA and MPA with the added polymers, PA, PMMA, PS and PVC, in all four donors are shown in Figure 14, Figure 15, Figure *16* and Figure 17, respectively. In all four donors, the same polymer is illustrated with the same colour. The control is the same for all the samples in each donor, however the percentage for the control for PNA and MPA are not the same as they are gated differently, as shown in Figure 7 and Figure **9**. Due to the limited number of data no statistical analysis was performed.

Figure 14. The percentage of neutrophils (A) and monocytes (B) aggregates in Donor 1. The x-axis shows the control and the different concentrations of microplastic in the samples. The y-axis displays the percentage of neutrophil-platelet aggregates (Figure 14a), and the percentage of monocyte-platelet aggregates (Figure 14b). Figures were created with GraphPad Prism. The percentage for the control

for Donor 1 were respectively 0.23% for the PNA and 0.32% for the MPA. PA had an increase in aggregates from the control to the C1, 0.33% for the neutrophiles and 0.4% for the monocytes. The other concentrations seemed to have a lower number of aggregates in a dose-response manner, whereas the lower the concentration the lower number of aggregates. This seemed to be the case for both neutrophils and monocytes. However, C5 in both populations demonstrated lower percentage of aggregates compared with the control. PMMA also seemed to have a formation of aggregates in a dose-response manner. An increase between the control and C1 were also seen here, both for neutrophils and monocytes. For the remaining polymers, PS and PVC, none of the concentration showed any increase in the formation of neither neutrophils- nor monocytes platelets aggregates. Regardless of the increases or decreases in the formation of aggregates, the same polymer seemed to follow the same pattern in both neutrophils and monocytes aggregates.
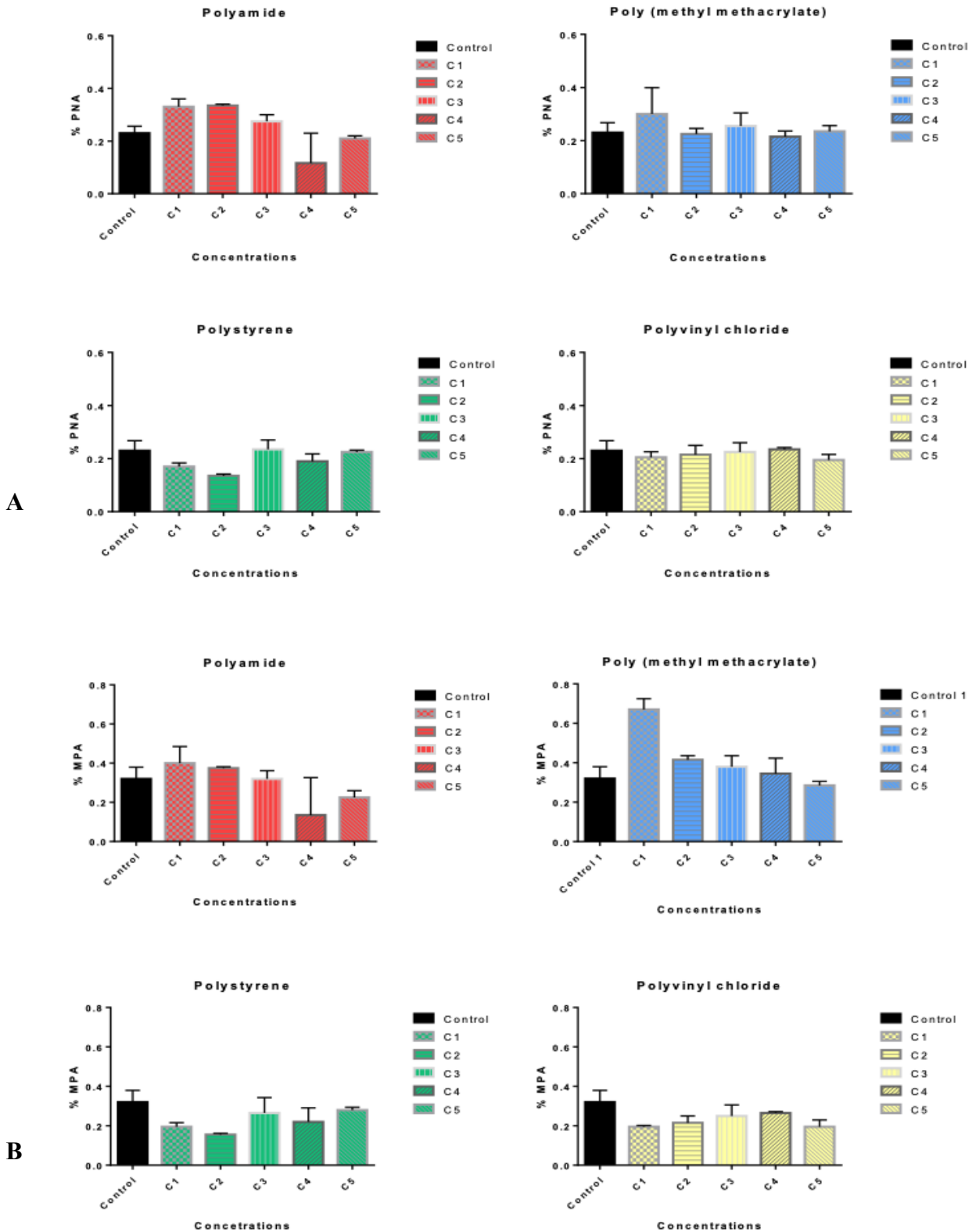
Figure 15. The percentage of neutrophil (A) and monocytes (B) aggregates in Donor 2. The x-axis shows the control and the different concentrations of microplastic in the samples. The y-axis displays the percentage of neutrophil-platelet aggregates (Figure 15a), and the percentage of monocyte-platelet aggregates (Figure 15b). Figures were created with GraphPad Prism.

The percentage for the control for Donor 2 were respectively 0.201% for the PNA and 0.368% for the MPA. PA had an increase in aggregates between the control and C1 for both neutrophils and monocytes, in a dose-response manner as seen in Donor 1. For PMMA this only applied to the monocytes, as all the concentrations were lower than the control for the neutrophils. PS only showed an increase in aggregates in the second highest concentration (C2) in the neutrophil population. For the monocytes, PS yielded various increases and decreases between the concentration. No dose-response pattern was observed here. For PVC in Donor 2, only the fourth concentration (C4) in the neutrophil aggregates demonstrated any increase from the control, as all the concentration for the monocytes were lower than the control.
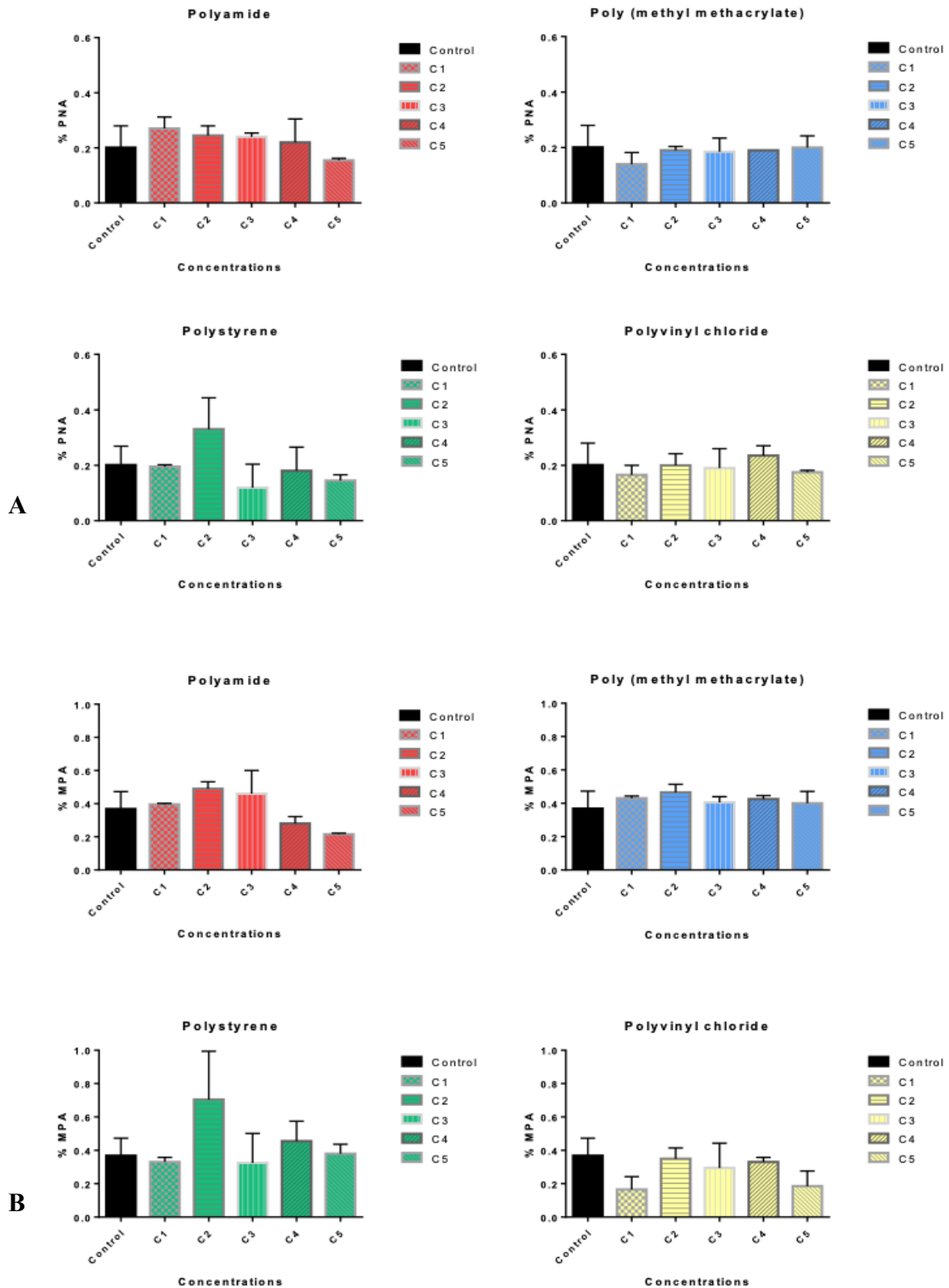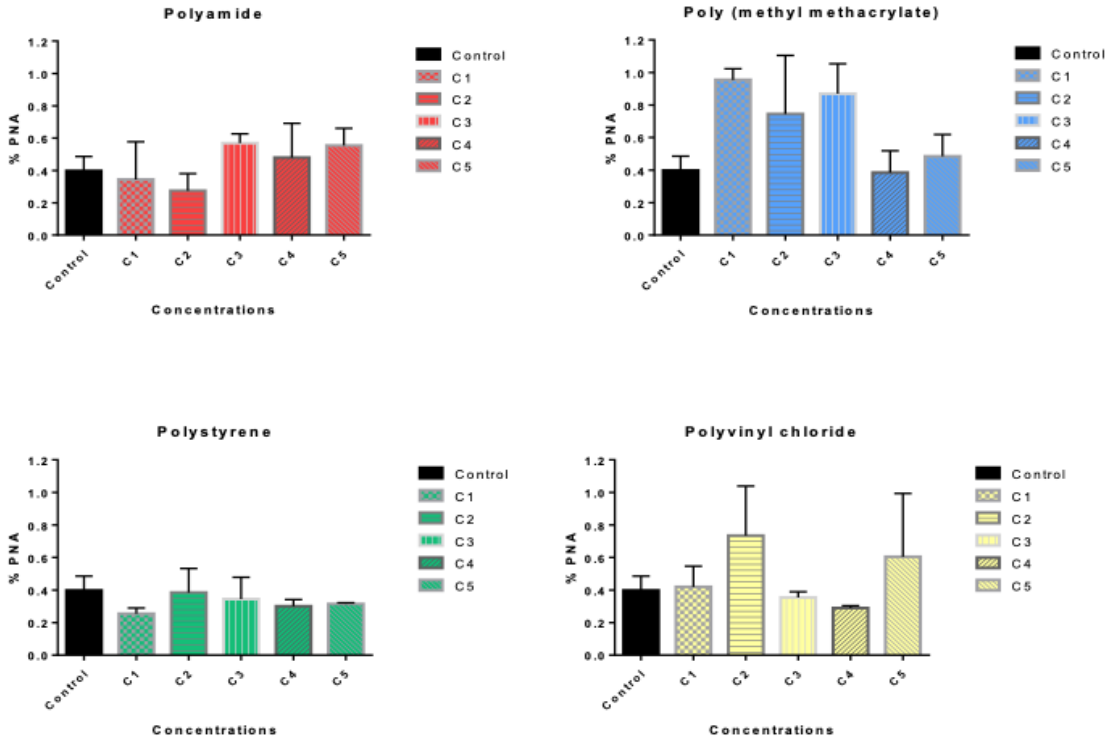
Figure 16. The percentage of neutrophils (A) and monocytes (B) aggregates in Donor 3. The x-axis shows the control and the different concentrations of microplastic in the samples. The y-axis displays the percentage of neutrophil-platelet aggregates (Figure 16a), and the percentage of monocyte-platelet aggregates (Figure 16b). Figures were created with GraphPad Prism.

The percentage for the control for Donor 3 were respectively 0.398% for the PNA and 0.496% for the MPA. PA in Donor 3 did not display any dose-response pattern, as seen in Donor 1 and Donor 2. C1 were lower than the control, both for the neutrophils (0.345%) and monocytes (0.4%). However, for the neutrophils C3, C4 and C5 were higher than the control but not in a dose-response manner. For the monocytes, both C1 and C2 had lower percentage of aggregates relative to the control. PMMA had the highest increase in neutrophils aggregates from to the control, with a percentage of 0.955%. For the monocytes a relatively large increase was also seen here, up to 0.845%, however the standard deviation here was somewhat high relative to the rest. All concentrations for PMMA in both neutrophiles and monocytes were higher than the control, except from C4 in the neutrophils. For PS, all concentrations were lower than the control. This applied to both neutrophils and monocytes, except from only C2 in the monocyte population. For PVC, in the neutrophil population C2 had the highest percentage of aggregates, with 0.735%, and C5 as the second with 0.605%. However, both of these had higher standard deviation compared to the rest. For the monocytes, C1 demonstrated lower percentage than the control (0.435%), with an increased formation in the C2 (0.875%), and the rest of the concentrations with decreasing percentage of aggregates. Control was higher than C5.
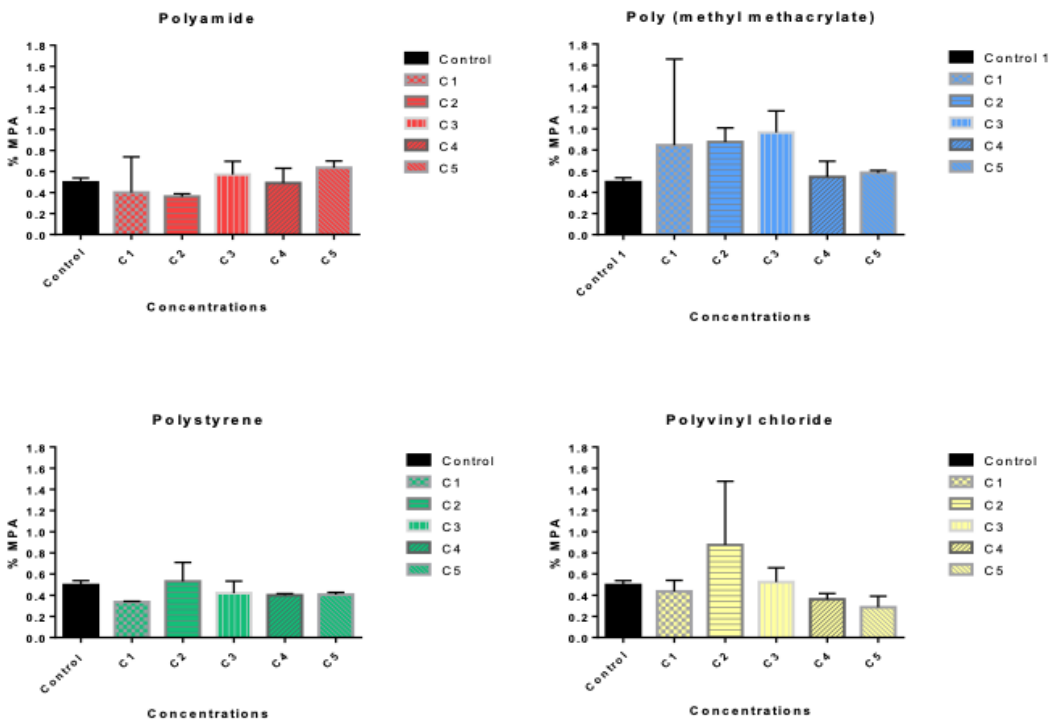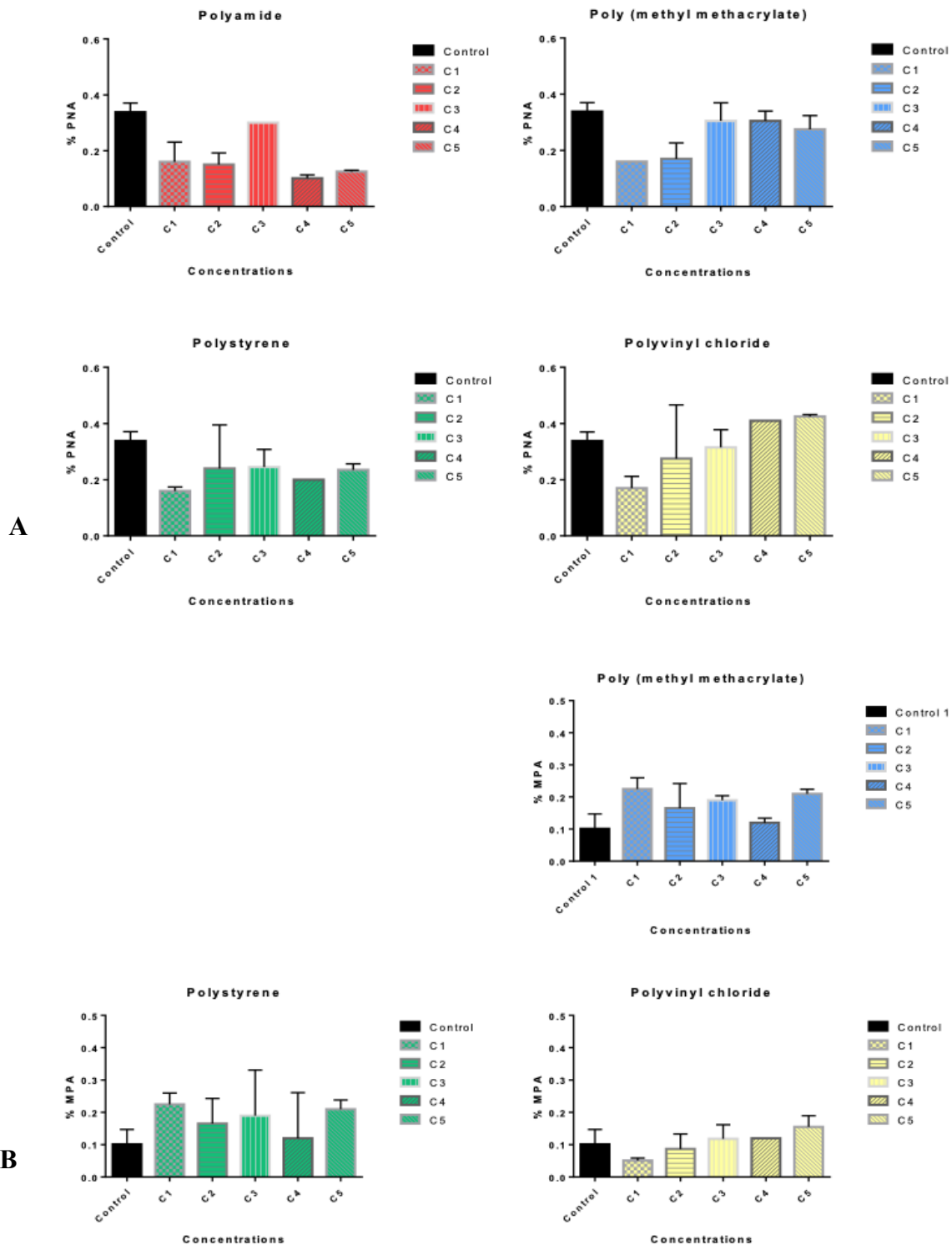
Figure 17. The percentage of neutrophils (A) and monocytes (B) aggregates in Donor 4. The x-axis shows the control and the different concentrations of microplastic in the samples. The y-axis displays the percentage of neutrophil-platelet aggregates (Figure 17a), and the percentage of monocyte-platelet aggregates (Figure 17b). The data from MPA for PA is missing, as explained in the Appendix 2. Figures were created with GraphPad Prism.

The percentage for the control for Donor 4 were respectively 0.338% for the PNA and 0.101% for the MPA. For the neutrophil population, the control was higher than all the samples with the highest concentration of MP for all polymers. All polymers demonstrated a decrease in PNA between the control and C1 among the neutrophils. For PA, none of the concentrations among the neutrophils demonstrated any increase in aggregates compared to the control. The data from the MPA for PA in Donor 4 was not included, as explained in the Appendix 2. For PMMA, all concentrations for the neutrophils were also lower than the control. For the monocytes on the other hand, all concentration were higher than the control, even though not in a dose-response manner. As for the neutrophils aggregates in both PA and PMMA, PS also demonstrated no increase in the formation of aggregates in Donor 4. For the monocytes, the different concentrations yielded various increases and decreases in the formation of aggregates. No dose-response pattern was observed here. As for the PVC, both the neutrophils and the monocytes aggregates demonstrated a reverse dose-response pattern whereas the lower concentration of MP the higher percentage of aggregates. This was the only polymer in which this reverse dose-response pattern was seen, however it was only observed in one of the donors.

5   Discussion

The purpose of this project was based on our hypothesize on whether humans can ingest MP through the consumption of contaminated food or drinks, and that MP can be recovered in the feces. Furthermore, some of the ingested MP may also be absorbed through the gastrointestinal epithelium and translocated into the human blood stream. In a previous study, NP particles were shown to interact with platelets and increase the risk of thrombosis (44). Thus, we carried out a two-fold study, analyzing chemically digested human feces samples using FTIR and a whole blood flow cytometry analysis of MP blood samples, spiked with different concentrations off individual polymers.

5.1 Study 1: Microplastic in human feces

5.1.1 Study 1: Discussion on results

We indeed showed the presence of MP particles in all fecal samples that were able to be analyzed with the FTIR. Our original plan was to assess whether one of the two dietary groups (seafood or meat) included in the study contained more MP than the other. For this reason, dietary data were also considered in order to hypothesize possible sources of MP. The FTIR analysis, however, only yielded a limit number of results. Nine samples resulted to have to high background noise to be analyzed for MP contamination. Reasons for this were most likely oxidized proteins present in the feces. The protocol was developed from rat feces, and such problems were not observed with rat feces neither in FTIR nor in py-GC/MS analysis. Thus, any statistical analysis was precluded due to the small sample size of the 10 remaining samples. We were not able to draw any firm conclusion whether one of the consumption groups posed more risk to ingest MP than other. To be honest, we also have to admit that we did not take into account other differences in the diet than meat or seafood intake or any differences in food packaging, as the original study was not designed for MP analyses. We cannot exclude the possibility that seafood is identified as a source of MP due to the large number of studies that investigated seafood, while other food sources have been less thorough investigated, but may also contain MP (13). Nevertheless, all these 10 samples contained MP, with concentrations ranging from 1 to 21 particles per sample. The three identified polymers were PP, PE and PS. This is not the first study assessing the presence of MP in human fecal samples (42, 43). In these two studies, the most abundant identified polymers were PP, PET and PS, which is also consistent with our results. PP and PE accounted for 50% of the plastic production globally in 2020, according to data from Plastic Europe (3). Both PS and PP are mainly used for food

packaging (3). However, we have not identified any PET particles. As PET is the most common polymer in plastic bottles, this could be due to the lower consumption of bottled water in Norway than in other countries, as bottled water has been demonstrated to contain more MP than tap water (13, 60). Unfortunately, we did not have information whether the participants used bottled water as the original study was not designed for MP analysis. In our study, PE was also quite abundant in the fecal samples, which is also used in food packaging and other household products (3). The results should be regarded as a pilot study; however, the results are in agreement with other studies on the occurrence of MP in human feces and urge for further analyses of MP sources and their fate in the human body. In order to assess the level of MP in all source materials and determine whether MP reside in the human body, more research is needed (43).

5.1.2 Study 1: Discussion on method

An optimal digestion protocol needs to be tailored for every different matrix. It is therefore, cumbersome to assess the MP contamination in every food product and the analysis of the feces may serve as a non-invasive matrix to estimate the ingestion of MP (13). It needs to be taken into account that MP in feces may not derive entirely from contaminated food products, as these particles may be released from different household products, such as food packaging, containers and kitchen sponges (61, 62). MP may also originate from inhaled air or dermal contact and then be excreted in bile into the gut and end up in the feces. One of the main challenges when studying MP in complex matrices, such as biological samples or food products, is the extraction process. Moreover, the smaller the particles, the more difficult it is to separate them from the matrix. This applies in particular to the smallest particles in the most challenging matrices, such as particles below 100 μm (26). As with any separation process, the separation itself requires that the particles or the solids differ in some way from each other. For plastics, it is often relied on their hydrophobic and density properties. Plastic tends to be more hydrophobic and less dense, especially compared to wet matrices (26).

Filtration is the simplest separation technique, which can categorize by particle size. However, its biggest limitation is the balance between its ability to capture the smallest particle and avoid clogging the filters. The filtration will only confirm the presence of the MP, and not the total numbers (26). Of 45 peer-reviewed papers on MP collection and separation published in a period between 2012 and 2018, 62% of the papers utilized chemical digestion as separation method. These were papers on fish, plankton, mussels and terrestrial animals (26). Matrix

digestion can be divided in three categories, respectively acidic-, enzymatic- and alkaline digestion (13). Acid- and base digestion are often being used to separate MP from biological matrices. Hydrogen peroxide (H_2O_2), nitric acid (HNO_3) and potassium peroxide (KOH) are usually applied to digest greasy matter. Despite being effective at removing organic matter, HNO_3 digestion may destroy the MPs due to the high temperatures and time it requires. Temperatures exceeding 80°C may also alter the MP surface properties (39). In the previous paper, Yan and coauthors used different chemicals for the feces extraction method, such as HNO_3 and Fenton´s reagent, which consisted of H_2O_2 and an iron catalyst solution, as a single agent is often not sufficient to digest complex organic compound, such as feces. Temperatures did also not exceed 60°C. The paper yielded high recovery rates, respectively 97.78%. Several digestion reagents need to be carried out together, in order to have a successfully result (39). However, while some polymers may be damaged by the strong chemicals, others seem to be more resistant to them such as PVC and PE. This can result in variety in the recovery results (26), and explain why only a limited numbers of polymers were found in our feces samples. In order to assess and optimize the different digestion steps, spiked controls are used to evaluate these approaches, considering the possible damage to the MP as well as to calculate recovery rates. Spiked controls, also referred to as positive controls, usually follows the same extraction steps as the real samples (26). A previous paper on the extraction of MP from human and chicken feces, evaluated the recovery rate of PS, PE and PVC particles (2 - 4 mm). They found the overall recovery rate to be 97.78% for both types of feces, whereas PE turned out to be the most resistant polymer with the highest recovery rate compared to PS and PVC (39). However, when assessing recovery rates, the size of the spiked MP should be chosen accordingly to the targeted size range investigated as smaller particles are more likely to adhere to the surface of the glass containers (63). Moreover, the possible chemical damage to smaller particles may increase due to the increasing surface to volume ratio at decreasing sizes (64). An efficient digestion method needs to simplify or eliminate background matrix, as well as to limit the possible damage to the MP (13).

However, another important thing to assess when analyzing the presence of MP in different samples is the possible background contamination. Given their ubiquitously presence, blank control samples are crucial aspects when analyzing particles as MPs. Blank controls are frequently performed to analyze any possible contamination. One must take into account that a small proportion in low concentration samples may be due to background contamination (26). Even though our quality control samples revealed no MP, indicating low contamination during

the processing, two of the human feces' samples did only contain 1 plastic particle. As such low concentration, it is difficult to exclude any contamination during the processing, e.g., form the surrounding, either airborne or from the equipment. As the feces sampling did not take MP contamination into account, it also may have been due to the sampling itself or the plastic containers. Also, the feces sampling kit consisted of PS, and may be a possible source of contamination. A further issue that must be considered is that the digestion steps prior to the FTIR analysis were not optimal, as they were not carried out in a laboratory suited for MP analysis. This could have contributed to MP contamination, even though we took all precautions as described in section 3.2.5, quality assurance and quality control (QA/QC).

5.2 Study 2: Microplastic and cell aggregation in human blood

The presence of MP in the diet and the feces have been demonstrated in our study and in several other (39, 41-43), suggesting with high certainty that we are ingesting MP through the diet. Of these ingested particles a small fraction could be taken up through the intestine and distributed to other organs with the blood circulation. The first proof of MP in human blood was published indeed in a paper in March 2022 (37). However, the effect of MP on platelet aggregation has not been investigated before. A study on NP on the other hand, has been performed by McGuinnes and coauthors (44). Given the presence of MP in blood we wanted to investigate whether different polymers in different concentrations in a small size range could influence the potential for platelet aggregate, as seen with the PS nanoparticles. Flow cytometry as an approach for evaluating platelet aggregation has become increasingly popular during the last couple of years. As determined by the binding of an activation-depended monoclonal antibody, whole blood flow cytometry can assess the activation state of circulating platelets without the addition of a exogenous platelet agonist (51). An increase in circulating MPA has been shown to be present in individuals who suffer from stable and acute coronary heart disease, with levels exceeding >15% (46). A study of PS nanoparticles with different surface modifications (23-330 nm) demonstrated their presence to affect blood coagulation. The impact of blood coagulation was assessed by the generation of thrombin, which are important in the cascade pathway forming a blood clot (38), while the McGuinnes paper identified NP of the same polymer to increase the formation of LPA (44), and thus increase the risk of thrombosis. Thrombosis is linked to cardiovascular disease (CVD), but also to many other conditions, e.g., infections, obesity and certain cancer types (47). As CVD is a major disease burden globally, it is important to acknowledge MP potential effect on human platelets. Vascular inflammation is the underlying mechanism for different CVDs, which are mainly initiated by leukocytes and platelets. Platelets are therefore an important mechanism in in the development of CVD (65, 66). As stated by World Health Organization (WHO), CVD is the leading cause of death globally, taking about 17.9 million (2019) lives each year (67).

5.2.1 Study 2: Discussion of results

The results from our flow cytometry analysis, were not consistent with the previous paper on NP particles and platelet aggregation (44). As the data material was limited, no statistical analysis was performed and no significance could be calculated, therefore no firm conclusion could be drawn. However, as there was no repeatedly distinct dose-response pattern for none

of the polymers, we cannot conclude that MP particles did have an effect on platelet aggregation, neither for neutrophils nor monocytes. We hypothesized that leukocyte-platelets aggregates would increase in a dose-response manner, whereas the higher the concentration of MP the higher the formation of aggregates. In some of the polymers, as often seen for the PS, the baseline reference was higher than any concentration containing MP. For this polymer at the given size and concentrations, it can be assumed that MP did not have any effect on the formation of aggregates. PMMA and PA, however, were the polymers that yielded the most distinct dose-response pattern, at least in some of the donors, respectively donor 1 and 2 for PA and donor 1 and 3 for PMMA. Due to the small number of participants, results are difficult to interpret. In healthy humans, neutrophils represent 40-70%, lymphocytes 20-45% and monocytes 2-10% in the leukocyte population. Thus, the levels of LPA are individual and therefore depends on the absolute number of leukocytes in patient blood (55). We did neither analyze the total number of leukocytes nor the specific cells in our samples. Therefore, differences in the baseline percentage between the donors may just reflect individual variations. As previous data suggest, the absorption and distribution of MP may be depended on particle size (1, 27, 28). McGuinnes et al. used nanoparticles with a size of 50 nm (44), while we included particles with a size range of 1-25 μm, thus exceeding the size in the former study by at least 20fold. Particle size may also limit translocation in the human body and can explain why we did not see any clear effect of the MP particles on human platelets. Oslakovic et al. observed larger effect on blood coagulation of the 57 nm animated NP compared to 330 nm, most likely due to the larger surface area-to-volume of the smallest particles (38). In exposure studies it is important to compare both concentrations and size, however studies often only report either concentration and size, making it hard to conclude whether particle concentration or particle size is of greatest concern (1). The majority of available data often use MP particles with a size range of 10-50 μm (37). Most exposure studies often used much higher concentration than what is found in the environment, as in our case. However, it still provides a useful indication on how MP may interact with organisms if they continue to increase in the environment (68).

Several factors can influence the effects of MP on platelets aggregation, such as size, shape, modifications/surface charge, type of polymer and concentration (1). We hypothesized that fragmented particles would have greater effect because of the rough edges and relatively larger surface. PA was pristine and thus spherical as it was purchased from GoodFellow, while

PMMA, PS and PVC were produced at the Institute of Marine Research in Bergen. Existing research on MP on terrestrial animals and humans have often been using one specific polymer, and in most cases PS (69). This polymer has several times been used in toxicological studies, often due to its density which allows easy suspension in aqueous media (5). PS can easily be generated into particles with precise size distribution, and is attachable to other molecules such as fluorescent dyes to ease detection (5). However, its use limits their relevance to environmental microplastic considering PE and PP are the dominant polymers, accounting for 50% of the plastic usage, and also confirmed in our fecal analysis study (3, 5). As the vast majority of existing literature are using PS, we wanted to test several polymers as different polymers may have different effect. As described earlier in the thesis, PS did not yield any increase in leukocyte-platelets aggregates, neither for neutrophils nor monocytes. However, it was PA and PMMA who showed greatest effects. As PA was the only spherical purchased polymer, we hypothesized lower effect compared to the other self-produced polymers which were non-spherical, with rough edges and larger surface. However, this was not the case here. As to why PA showed greater effect compared to PS and PVC is not clear,

A lot of research has also been done on pristine, manufactured microplastic. MP and NP may undergo several steps prior to ingestion by humans, such as weathering, aging, leaching of additives and the formation of a protein corona. This means that the various steps can change the properties and shape, and thus influence their fate in biological systems (5, 28).

5.2.2 Study 2: Discussion of method

Due to the limited number of donor blood samples, we were not able to perform any statistical analysis or conclude whether one polymer increased the risk of aggregation more than others. In order to perform any statistical analysis or to draw any conclusions, the data material should have been more extensive. With including several more donors, we would have gained more information on how the different polymers unfolded itself in the blood. However, the small sample size is due to the cumbersome technical preparation prior to the flow cytometry analysis and also costs. As blood is a very vulnerable matrix, the analysis should be done immediately after blood drawing. As we tested four different polymers with five concentrations each, in addition to control and unstained samples, all in duplicates, we had to prepare circa 60 samples for each donor. Therefore, we were only able to do one donor at a time. Since this type of analysis has not been done before, and was both cumbersome and expensive, and due to limited access to both the core facility and skilled personnel, it was not possible to include more

volunteers, as part of the master thesis. Thus, the results should be interpreted as preliminary. However, given the lack of a dose-dependent response, and the very small effects, seen in all 4 volunteers, it can be questioned whether inclusion of a larger size would have changed the conclusion and whether it is worth to spend more time and money on the analysis of LPA in fresh blood with MP.

As the formation and measurement of LPA are very sensitive to environmental factors, several factors needed to be considered prior to the flow cytometry analyses. The blood samples were treated in a standardized matter to reduce variability, including anticoagulant, time from blood drawing to analysis, and the different steps involved int the sample preparation for the flow cytometry. Sample preparation are, therefore, critical steps in obtaining comparable results. Type of anticoagulant, erythrocyte lysis, storage and temperature will affect the outcome (55). A paper by Harding and coauthors evaluated different methodological considerations in flow cytometry analysis of MPAs (53). Due to the high sensitivity of the assay, reliable methods are crucial to ensure stable outcomes. Harding et al. studied the effect of different handling- and processing techniques on the formation of MPA. They studied different anticoagulants, namely heparin, PPACK, sodium citrate and EDTA, and found heparin to increase the formation of MPA compared to the other anticoagulants (53). Another review stated that EDTA should not be used as an anticoagulant for LPA analysis, as an in vitro dissociation of platelets from leukocytes can be caused by EDTA (50). Harding et al. also looked into the effect of time delay prior to antibody staining, with intervals ranging from 0, 10, 20, 30 and 60 minutes between the blood sampling and the labelling. They saw an increase in aggregates in a time-depended manner, whereas the longer the interval the higher percentage of MPA (53). They also evaluated the effect of time delay of the fixed samples prior to flow cytometry analysis, at the following time points: 4, 8, 12 and 24 hours. This did not affect the formation of aggregates (53). As both Heparin as an anticoagulant and long interval between sampling and antibody labelling has shown to increase the formation of aggregates, the use should be reduced to a minimum if it is causing spontaneous aggregation. We followed the recommendations by using citrate anticoagulated blood and preparation of blood was finished within 1 hour sampling.

In addition, the flow rates of the flow cytometry are an important aspect to consider when performing flow cytometry analyses. As the concentrations of platelets in human blood are much higher than monocytes, non-adherent events may occur especially if the sample is run on high flow rates. This could lead to events passing through the laser near each other at the same time and may not be distinguishable. If so, it will be recorded as a MPA event. Therefore, it is

important to run the samples on low flow rates (46). The coincidence events have proven to exceed 30% in some studies and may explain some of the variability of MPA in the reported results (70). This was also considered in our analysis, and every sample were run on low flow rate. The different emission spectra of each fluorochrome can overlap with other, and this can lead to difficulty in resolving the different signal, however with proper compensation this should be small as long as the flow cytometry panel has been set up properly. In flow cytometry there is a limit to the number of fluorochromes that can be used at the same time, and this has to be taken into consideration when setting up a panel (52). As the compensation were performed, this should not be a limitation in our study.

As all this was taken into account when performing the flow cytometry analyses, the results are most likely reflecting little or no effect of MP on platelet aggregation.

6   Conclusion

In this pilot study, we were able to demonstrate the occurrence of MP in 10 of 10 fecal samples that yielded a valid result from thee FTIR analysis, thus clearly demonstrating the presence of MP in human feces. Improvements of the preparation method, however, is required, as 9 samples were not able to be analyzed by FTIR due to the high background noise. The presence of MP in human fecal samples makes the ingestion of MP with food and drinks very likely and should give a cause of concern regarding food safety.

MP of different polymer added to fresh blood samples in different concentrations did not lead to the formation of platelet-leukocyte aggregates in a dose-dependent manner in 4 different donors, neither for neutrophils nor the monocytes. Even if MP has now been demonstrated also in human blood, our results suggest that platelet activation is not a major pathway for harm caused by MP in blood. Further analyses and experiments are required to investigate the role of MP on other platelet functions, interactions with the protein coagulation pathway and other pathways like inflammation. In order to address the possible sources and negative effects, more research is needed. As there is a current lack of health risk assessment for MP exposure, it is not clear whether MP actually pose a public health risk. Thus, future perspectives should address how MP unfold itself in biological systems, in terms of different size, shape, polymer and modification. As MP is found in several matrices it also should be standardized sample preparation for different matrixes, that simplify further analyses, such as FTIR and py-GS/MS. Without reference material, the developement of monitoring programs and frameworks for risk assessment is challenging.

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8 Appendix

8.1 Appendix 1: Optimization steps feces protocol

The feces digestion protocol used in this study has been adapted from a rat feces protocol, which included three digestion steps: (i) acidic digestion, (ii) alkaline digestion and (iii) enzymatic digestion. As human´s and rat`s intake of fiber is highly different, the cellulose content in human feces is expected to be lower than in rat feces. Therefore, we hypothesize that the enzymatic digestion step would not be needed for human feces.

The overall aim was to establish a digestion protocol able to digest different type of human feces. Since the feces content is dependent on the diet of an individual, we collected different feces samples after a three-days diet rich in either chicken, red meat or fish. Moreover, a sample of feces from a two-years old was also included in the testing protocol. Initially, 0.1 g or 0.3 g of fecal material for every group (namely chicken, fish, beef, and toddler) were processed as follow:

- **Day 1**: 0.1 g (F.1, C.1, B.1 and T.1) and 0.3 g (F.2, C.2, B.2 and T.2) of feces were weighted and placed in glass container. 30 ml of the mixture H_2O_2 15% and HNO3 5% was added. The samples were covered in aluminum foil and incubated for 20-24 hours at 37°C and 140 rpm.

- **Day 2**: The samples were filtered through PTFE (5 μm pore size, Sigma-Aldrich) using a vacuum pump. For the 0.1 g samples the filtration process was fast. For the 0.3 g was slow and the washing steps took longer. All samples were washed with pre-filtered 0.01% (m/m) Tween®-20, ethanol-water (1:1, v/v) and then water. The filter was then placed in the same glass container used previously and 30 ml of KOH 10% (m/m) were added to each sample. Sample were covered with aluminum foil and incubated again under the same conditions.

- **Day 3**: The samples were filtered through Whatman GF/C (1.2 μm pore size, Sigma-Aldrich). The glass container and the samples were washed with 0.01% (m/m) Tween®-20, ethanol-water (1:1, v/v) and water. Then few drops of KOH 10% were added directly on the filter. The process of filtration was fast for samples with 0.1 g of material (except for the toddler group). However, the filtration for 0.3 g samples was impossible (Figure S1).
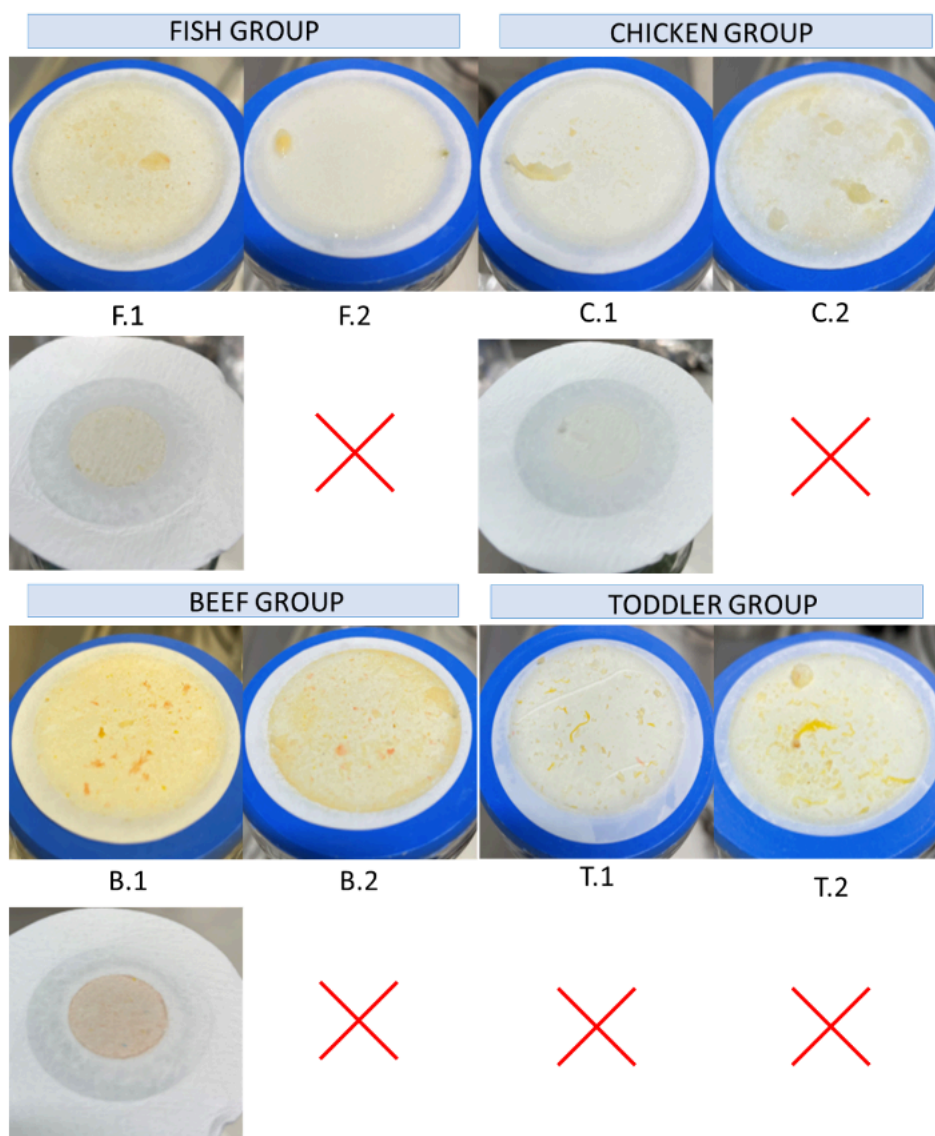
Figure S1. Filters obtained from digestion of feces samples from human following a three-days diet rich in either fish, chicken or beef or feces from a toddler. First step: remnant of feces material on PTFE filters. Second step: remnant of feces on Whatman GF/C filters. 0.1 g of feces (F.1, C.1, B.1 and T.1) and 0.3 g of feces (F.2, C.2, B.2 and T.2). The red cross indicate that the filtration was not possible.

However, the use of Whatman GF/C filters was not recommended for the following FTIR analysis as it would have given background signals. Therefore, the personnel at NORCE suggested to use PTFE filters in both the filtration steps. PTFE filters have a pore size of 5 μm while Whatman GF/C have a pore size of 1.2 μm. The FTIR limit of detection was around 15-20 μm and using 5 μm filters would be sufficient. For this reason, the same protocol was performed with 0.5 g of fecal material for the fish, chicken and beef groups with good filtration

rates due to the bigger pore size of the filters used in the second filtration step (5 μm versus 1.2 μm). Therefore, this protocol was chosen for the experiment performed in this thesis work.

8.2 Appendix 2: Flow cytometry

Flow cytometry as a suitable method to characterize microplastic in solution:

In the development of a reliable protocol, we wanted to identify and investigate the properties of different MP polymer before the *in vitro* toxicity experiment. Due to the specific autofluorescence given by some plastic polymers it is possible to detect them through flow cytometry analysis without the use of specific dyes. Therefore, we performed:

1. Flow cytometry analysis of different MP polymers in PBS in order to set the gate of one particular polymer

2. Flow cytometry analysis of the different polymers in EDTA human blood (including PA, PS and PVC used in the *in vitro* toxicity study) to see if it was possible to distinguish MP and blood cells.

3. Flow cytometry analysis of different concentration of MP in order to assess the lowest concentration detectable with flow cytometry analysis

Upon testing different MP dispersed in PBS or mixed with human blood it was observed that the BV510-a channel (brilliant violet 510) gave better autofluorescence results. In Figure S2 below, it can be seen the highest concentration (circa 150 μg/ml) of MP in both PBS and human blood. The different polymers have a different gate based on their size and complexity. Only in the case of PA and PEEK it is possible to distinguish between the gates of blood cells and MP particles. While for PS and PVC the gate of the microplastic particles overlapped the one of the blood cells, making it impossible to distinguish between MP particles and blood cells. Therefore, based on this preliminary results PS and PEEK were suitable for analysis with flow cytometry.

The lowest concentration of MP (circa 1 μg/ml) was detected only when dispersed in PBS, as the number of blood cells was much higher and therefore the threshold was reached before enough MP particles were detected in the samples.

This could be a promising method to detect microplastic in liquid samples. However, it was not part of the aim of the present thesis it will not be further discussed here.
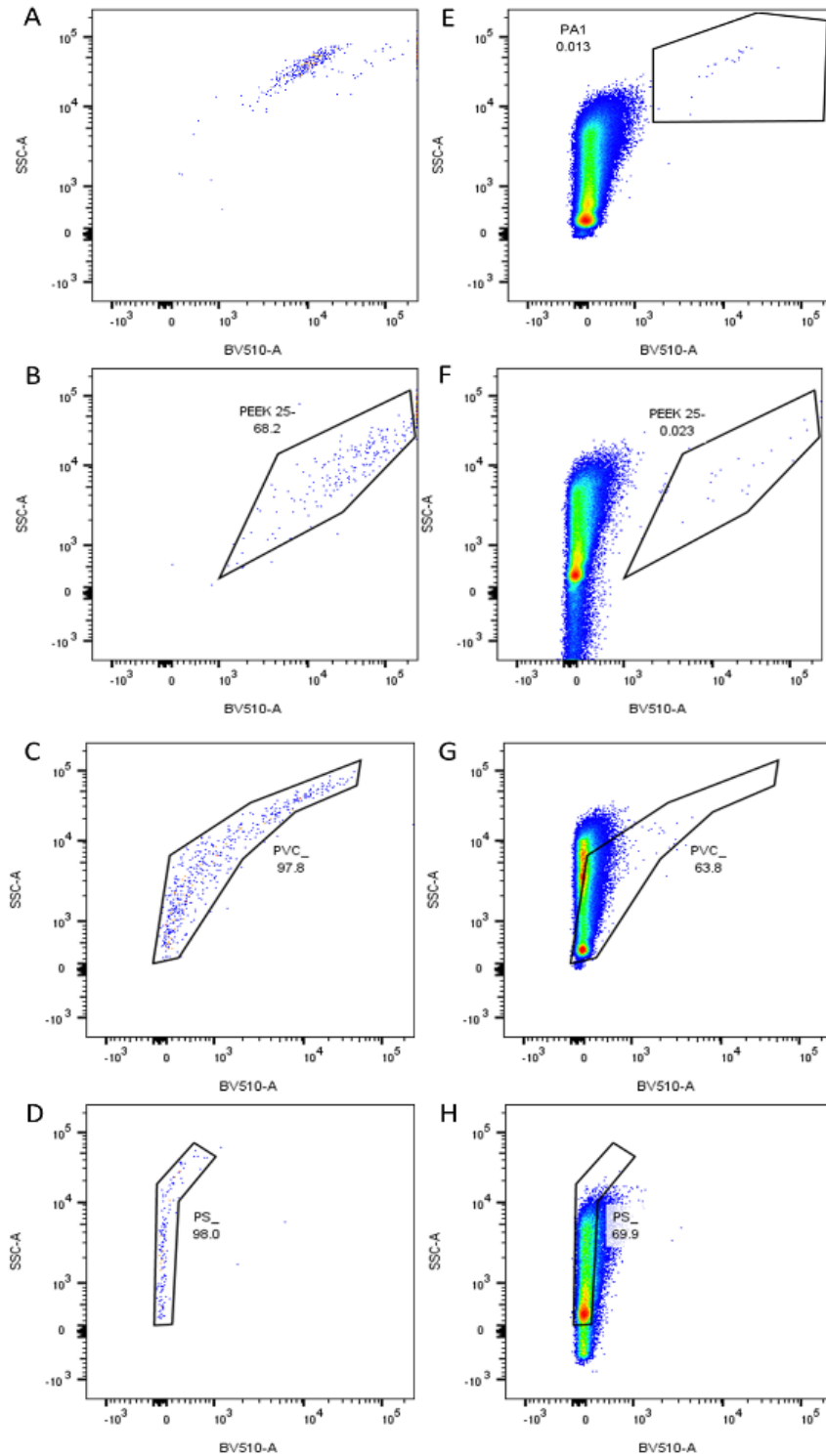
Figure S2. Example of the highest concentration of microplastic in PBS and in human blood. The figure shows the autofluorescence in the BV510-a channel for the highest concentration of PA, PEEK, PVC and PS dispersed in PBS (A, B, C, D) and in human blood (E, F, G, H). Figure created with FlowJo (version 10.8, BD Biosciences).

Excluded sample

In the *in vitro* toxicity study, we analyzed the percentage of both monocyte- and neutrophile-platelet aggregates in human blood upon exposure to four different polymers, namely PA, PMMA, PS and PVC in four donors. In order to identify monocyte, neutrophile and platelet populations, three specific antibodies were applied CD14-PE, CD16-APC and CD42a-FITC respectively. However, when analyzing the monocyte-platelet aggregates for PA in donor 4 we noticed an error in the sample. It did not show any fluorescence signal for both monocyte and platelets (Figure S3). This could be due to a mistake in the preparation of the samples. However, when the same sample was analyzed for neutrophile-platelet aggregates the signal was normal, suggesting that the problem was not related to the antibodies used as they were added together as a cocktail.
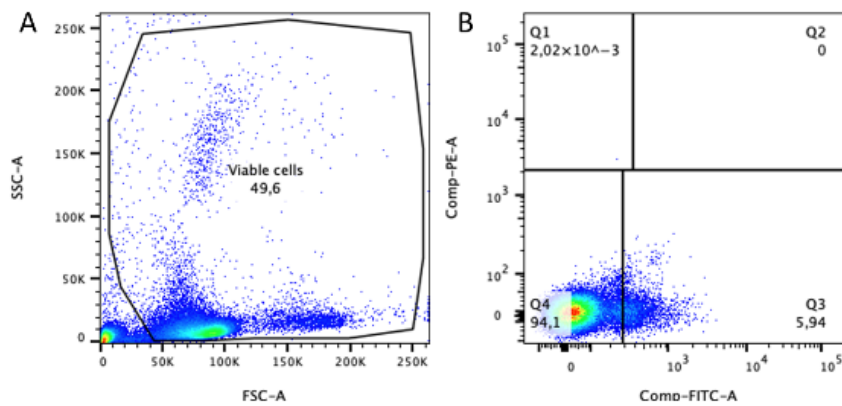

Figure S3. Monocyte-platelet aggregates from donor 4 exposed to polyamide. In figure S3 is clearly visible the absence of fluorescence signal for monocytes and platelets. Figure created in FlowJo (version 10.8, BD Biosciences).

8.3 Appendix 3: Materials and suppliers

Table S1. List of instruments used in the study

Instrument	Application	Supplier
Vacuum filtration	Filtration	Millipore
Nicolet™ iN10 MX Infrared Imaging Microscope	ATR-FTIR analysis	Thermo Scientific
6-digit electronic semi-microbalance R 180D	Milligram weight	Sartorius
TurboVap 500	Evaporation	Caliper Life Science
Incubator Hood TH 30	Sample preparation	Edmund Bühler GmbH
BD FACSCanto™ II digital FCM analyzer	Flow cytometry analysis	BD Biosciences

Table S2. List of equipment used in the study

Equipment	Application	Supplier
Mitex™ Membrane filter (PTFE)	Filtration	Sigma Aldrich
Whatman™ GF/C	Filtration	Sigma Aldrich
Whatman™ Anodisc inorganic filter membrane	Filtration	Sigma Aldrich
Glass wear	Sample preparation	VWR
Glass wear	Filtration equipment	Millipore
Glass pipette	Pipette	VWR
Eppendorf pipette	Pipette	VWR
Sodium citrate tube	Blood tube	BD Biosciences

Table S3. List of chemicals, reagents and antibodies used in the study

Name	Application	Supplier
Nitric acid 5% (HNO_3)	Fecal samples preparation	VWR
Potassium hydroxide 10% (KOH)	Fecal samples preparation	Sigma Aldrich
Hydrogen peroxide 15% (H_2O_2)	Fecal samples preparation	VWR
Ethanol (C_2H_6O)	Fecal samples preparation	Antibac
Hepes buffer	Blood samples preparation	Self-made
BD FACS™ lysing Solution 10X Concentrate	Blood samples preparation	BD Biosciences
CST calibration beads	Flow cytometry analyses preparation	BD Biosciences
UltraComp eBeads™ Compensation Beads	Flow cytometry analyses preparation	Thermo Fisher scientific
CD16 Monoclonal Antibody (eBioCB16 (CB16)), APC, eBioscience™	Blood samples staining	Thermo Fisher scientific
CD14 Monoclonal Antibody (61D3), PE, eBioscience™	Blood samples staining	Thermo Fisher scientific
CD42a Monoclonal Antibody (GR-P), FITC, eBioscience™	Blood samples staining	Thermo Fisher scientific

Table S4. List of plastic material used in both feces and blood study

Polymer	Size	Application	Supplier
Polyamide (PA)	$< 25\mu$m	Blood toxicity	GoodFellow
Polystyrene (PS)	$< 25\mu$m	Blood toxicity	Sigma-Aldrich
Polyvinylchloride (PVC)	$< 25\mu$m	Blood toxicity	Sigma-Aldrich
Poly (methyl methacrylate) (PMMA)	$< 25\mu$m	Blood toxicity	Sigma-Aldrich

Table S5. List of software used in the study

Program	Application	Supplier
OMNIC Picta	FTIR spectra analysis	ThermoFisher scientific
OMNIC Spectra	FTIR spectra analysis	ThermoFisher scientific
ThermoFisher Connect	FTIR spectra analysis	ThermoFisher scientific
Excel	Calculations, graphs	Microsoft
PowerPoint	Figures	Microsoft
FACSDiva v6.1	Flow cytometry analysis	BD Biosciences
FlowJo™ v10.8	Flow cytometry data analysis	BD Biosciences
GraphPad Prism	Graphs	Dotmatics