MethodsX 10 (2023) 102057

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

# Fast-screening flow cytometry method for detecting nanoplastics in human peripheral blood

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## ARTICLE INFO

*Method name:*
Nanocytometry

*Keywords:*
Nanoplastics
Plastic pollution
Blood
Flow cytometry
Live Cell Imaging
Nile Red

## ABSTRACT

Plastic pollution is a global problem. Animals and humans can ingest and inhale plastic particles, with uncertain health consequences. Nanoplastics (NPs) are particles ranging from 1 nm to 1000 nm that result from the erosion or breakage of larger plastic debris, and can be highly polydisperse in physical properties and heterogeneous in composition. Potential effects of NPs exposure may be associated with alterations in the xenobiotic metabolism, nutrients absorption, energy metabolism, cytotoxicity, and behavior. In humans, no data on NPs absorptions has been reported previously. Given that their detection relies significantly on environmental exposure, we have prospectively studied the presence of NPs in human peripheral blood (PB). Specifically, we have used fluorescence techniques and nanocytometry, together with the staining of the lipophilic dye Nile Red (NR), to demonstrate that NPs can be accurately detected using flow cytometry.

- Potential effects of nanoplastics exposure.
- Fluorescence techniques and nanocytometry.
- Accurate detection using flow cytometry.

## Specifications table

| Subject area | Environmental Science |
|---|---|
| More specific subject area: | *Plastic pollution* |
| Name of your method: | *Nanocytometry* |
| Name and reference of original method: | *NA* |
| Resource availability: | http://flowrepository.org/id/FR-FCM-Z5M6 |

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https://doi.org/10.1016/j.mex.2023.102057
Available online 6 February 2023

## Method details

Plastics are versatile materials widely used for many applications, especially in packaging, because of their ideal characteristics of bio-inertness, ductility, transparency, low cost, and lightweight. In the last years plastic accumulation and pollution have become a global issue and its reduction and management has arisen as a public concern. The most prevalent form of plastics are resins and fibers, prime examples being polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyethylene terephthalate (PET), and polyurethanes (PUR) resins; along with PP&A fibers (polyester, polyamide, and acrylic fibers). The global production of such resins and fibers increased from 2 megatonnes (Mt) in 1950 to 390.7 Mt in 2021 [11,31].

The major polymer types are non-biodegradable and degrade mainly by photo-degradation and thermo-oxidative degradation [1,23]. Many environmental pollutants are plastic debris that can be found in different sizes and states of degradation. Microplastics (MPs) are particles of less than 5 mm in diameter [6] that can origin either primarily from industrial and domestic use, such as cosmetic or pharmaceutical products, or secondarily from the weathering of larger plastics by biological degradation, photo-degradation, chemical deposition and physical fragmentation, such as tire abrasion and synthetic textiles [1,6]. Furthermore, nanoplastic (NPs) particles range from 1 nm to 1000 nm [12] and can derive from microplastic fragmentation [7,24], plastic usage degradation or manufacturing [12].

The detrimental consequences that these persistent plastic residues cause at the macrometric scale are obvious, ranging from physical traps [17,33] and toxicity [22,29] to global ecological impacts on ecosystems. However, the extent of the MPs and NPs persistence and their potential impact in health has not been studied in depth. Nevertheless, every year more studies about their effects on many organisms are being published; for a recent review see [4]. Depending on its shape, small plastic particles may possess a large surface area and increased hydrophobicity, with an increased ability to adsorb heavy metals, organic pollutants and other pathogenic molecules, which also can lead to an increased toxicity [18,44].

MPs and NPs effects have been described as toxic for many organisms, causing neurotoxicity, reproductive abnormalities, and endocrine system disruption, among other effects [4,40]. Regarding humans, plastic uptake is mainly associated with ingestion of polluted food and water, and to inhalation. It is theorized that MPs and NPs can be absorbed by the intestine and lungs [41] and accumulated in other tissues. For instance, in mice, it is known that plastic microspheres are absorbed by the microfold cells of the Peyer's patch and can be disseminated within macrophages to the mesenteric lymph nodes, blood circulation and spleen [8]. The toxic effects in humans still remain largely elusive. However, constant exposure to MPs and NPs may lead to potential serious health effects, such as those including genotoxic damage and oxidative stress in lymphocytes and macrophages [5,21,34], and changes in erythrocyte morphology and membrane asymmetry [19]. Recent publications in mice demonstrate, in vivo, a significant immune activation after exposure to inhaled polystyrene NPs, leading to pulmonary toxicity [42], and behavioral alterations [16]. Other studies have reported plastic exposure in humans, such as in feces [36], blood [25], or placenta [32] and meconium [3]. NPs are transferred to their offspring as it has been demonstrated in zebrafish [30], and can diffuse to immunoprivileged tissues, as previously demonstrated [43]. Specifically, previous work assessing the levels of MPs or NPs in human peripheral blood has been conducted by Fourier Transform Infrared (FT-IR) spectrometry [27], by Scanning Electron Microscopy coupled with an Energy Dispersion Detector (SEM-EDX) [10], and by double shot pyrolysis-gas chromatography/mass spectrometry [25].

In this study, we focus on a rapid and highly reproducible strategy to measure the NPs accumulation in human peripheral blood. We have developed a simple and robust method for flow cytometry based on the fluorescent staining of nile red. Nile red (NR), a red phenoxazone dye that binds to the surface of plastics and neutral lipids, was used to determine the presence in blood of the most common plastics: low density polyethylene, polystyrene, polyethylene terephthalate, and polyamide. NR was first described as staining cytoplasmatic lipid droplets [15] and was used to detect MPs in the marine environment using flow cytometry [2]. Other studies used fluorescence microscopy [9,26,37], ultraviolet light (UV) photography [38] and photoluminescence (PL) spectroscopy [20]. NR has a strong fluorescence in hydrophobic environments and its excitation/emission maxima are 552/636 nm in a polar solvent. NR is solvatochromic and shifts to shorter wavelengths with decreasing solvent polarity, meaning that it exhibits a spectral blue shift in non-polar solvents [14]. Importantly, NR is reported to successfully stain some of the most abundant microplastics found in surface waters, such as PE, PS, PP, PVC and PUR resins and polyamide fibers, by microscopic techniques and FT-IR identification [9,35,37].

In the present work, we have used fluorescence techniques and nanocytometry together with the staining of the lipophilic dye NR, to demonstrate that plastics can accurately be detected by flow cytometry.

### *Human samples*

Peripheral blood samples from healthy donors and patients were collected in EDTA tubes and stored at room temperature in agitation. Samples from healthy donors (HD, $n = 37$) and newborns (NB, $n = 36$) were obtained from the Blood Bank - Banc de Sang i Teixits - at the Hospital Universitari Germans Trias i Pujol (HUGTIP). Multiple myeloma (MM, $n = 28$), acute myelogenous leukemia (AML, $n = 46$), acute lymphoblastic leukemia (ALL, $n = 26$), chronic lymphoid leukemia (CLL, $n = 16$), and non-small cell lung cancer (LC, $n = 16$) patients were attended at HUGTIP. Idiopathic nephrotic syndrome (INS, $n = 9$) patients were attended at Hospital Sant Joan de Deu. Patients were recruited at different disease timepoints, which ranged from diagnosis and treatment to post-hematopoietic transplantation. Patients with type 1 diabetes (T1D), from diagnosis to 12 months of disease evolution ($n = 10$), were recruited at the Pediatric Section at HUGTIP and Parc Taulí University Hospital (Sabadell, Spain). All patients fulfilled the American Diabetes Association classification criteria for T1D.

*Mice samples*

Wild-type non-obese diabetic (NOD) mice were bred in the center for Comparative Medicine and Bioimage (Badalona, Spain) under specific-pathogen-free (SPF) conditions. Mice were in a temperature- and humidity-controlled room with 12 h light/12 h dark cycle, provided with standard chow diet (Teklad Global 14% Protein Rodent Diet, Envigo, Sant Feliu de Codines, Spain) and water ad libitum. Blood samples were obtained by intracardiac puncture (300 μl). Normoglycemic 7-week-old females ($n$ = 7) were included in the study.

*Sample preparation and flow cytometry*

All samples were manipulated in a BSL2+ environment. Briefly, 20 μl of PB were incubated in 1% aqueous potassium hydroxide (KOH) (Merck) (w/v, 1000 μL final volume) in a thermal block at 60 ˚C for a minimum of 10 days, to degrade the organic matter. Based on high chemical resistance and high thermal resistance, 1.5 mL Eppendorf Safe-Lock® tubes were used in this study. After incubation, three replicates were prepared for sample, by diluting 20 μL of digested sample in deionized water (1000 μL final volume) and stained with 2 μL of NR (Sigma-Aldrich; 0.1 mg/ml stock solution). After 15 min incubation at room temperature and light protected, samples were acquired on the Attune™ NxT Flow Cytometer (Thermo Fisher) at the lowest possible sample rate (12.5 μL/min), with the H-pulse parameter and collecting violet-side scattering using the Attune™ NxT Violet Side-Scatter Filter Kit. The resolution of nanoparticles detection is improved with the Violet Side Scatter (VSSC) since the violet laser (405 nm) has a lower wavelength than the blue laser (488 nm), thus yielding smaller coefficients of variation and a greater separation index [45]. NR was excited at 561 nm and its emission was collected with a 585/16 BP filter in the YL1 detector. An exhaustive rinse with filtered deionized water was performed between each sample to prevent carry over. In order to avoid potential environment contaminations, plastics measurements were always done in triplicate and a quadruplicate was added when one of the results had a discrepancy. Liquid solutions and deionized water used in this study were always monitored for potential issues with undesired plastic contamination. In addition, all the experiments were performed after carefully purging the fluidic system on a dedicated flow cytometer. Since all blood specimens were diluted for sample preparation and acquisition, a factor of x250 must be applied to convert the raw data (FCS files) into blood concentration units. Additionally, and before sample acquisition, a deep flow cell cleaning was performed with Hellmanex® III (Attune NxT Flow Cell Cleaning Solution, Cat# A43635), a liquid alkaline concentrate for highly effective cleaning of cuvettes made of glass and quartz glass and other sensitive optical parts. Alternatively, Contrad® 70 (Beckman Coulter, Ref# 81,911), a liquid detergent concentrate phosphate-free, chlorine-free, and biodegradable can be used to effective cleaning cuvettes and tubing. The small particle kit consisting of 0.2 μm, 0.5 μm, 0.8 μm beads (Bangs Laboratories, Inc) was used for calibration, which was generously provided by the Head of the Flow Cytometry and Cell Sorting Core Facility at Germans Trias i Pujol Research Institute (IGTP).

*Live-Cell analysis*

The IncuCyte® SX5 Live-Cell Analysis Instrument (Sartorius Stedim) was used as a real-time system to study phagocytic activity up to two days in a cell culture incubator. Fluorescent green microspheres (Count Check Beads green, Sysmex) were added for the rapid and accurate detection and quantification of in vitro phagocytosis. Briefly, peripheral blood was lysed with BD Pharm Lyse Lysing Buffer 1x, an ammonium chloride-based reagent for erythrocyte lysis. Following red cell lysis, cells were pelleted by centrifugation, the lysing reagent was removed, and the cells resuspended in RPMI medium with 10% inactivated FBS (Biowest), 2% L -glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin (Biowest), preventing inhibition of phagocytosis by removing residual EDTA. Cells were permanently maintained in a humidified incubator at 37 ˚C with 5% $CO_2$.

*Statistics*

All measurements were performed in triplicate and a quadruplicate was added when one of the results had discrepancy. Graphs were generated with Prism software (v.9). Median (m) and range (r) values were calculated for a total of 196 human subjects and 7 mice included in this study. Absolute counting of NPs found in human and mice samples were compared by using the Mann-Whitney test. A p-value $< 0.05$ was considered statistically significant.

*Quality control*

We first monitored the potential impact of plastic use in sample contamination. Blood collection tubes are made of durable polyethylene terephthalate (PET), one of the most recycled thermoplastics. Importantly, Eppendorf Safe-Lock® tubes are specially designed for quality control in food and beverage and water supply, as well as in environmental monitoring, and were chosen based on a reduced probability of contamination, high chemical resistance and high thermal resistance. Deionized water and aqueous KOH were always tested to ensure the lowest possible signal of NR fluorescence. Moreover, potential variation of NR fluorescence obtained from different stock solutions used over time, were always monitored by using blank beads, stained in the presence of NR for 15 min at room temperature and light protected, to ensure fluorescence calibration (Supplementary Figure 1). All the steps needed to study plastic accumulation in human blood were performed under a BSL2+ environment to minimize potential airborne plastic particles contamination. Plastic measurements obtained from deionized water, EDTA tubes and aqueous KOH were consistently less than 71 events/μL (Fig. 1 and Supplementary Figure 2). In order to ensure efficient staining, all measurements were performed in triplicate

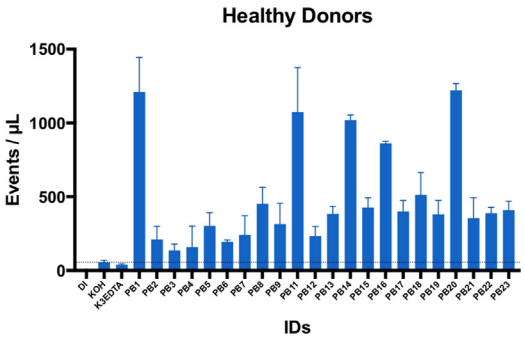


**Fig. 1.** Consecutive analysis of nanoplastics in human peripheral blood obtained from healthy donors. The background contribution was assessed in deionized water (DI), aqueous potassium hydroxide (KOH) and deionized water diluted in K3EDTA tubes. Plastic measurements obtained from deionized water, EDTA tubes and aqueous KOH were consistently less than 71 events/µL. Peripheral blood analysis of $n = 23$ consecutive donors is shown here and compared with the background signal. All measurements were performed in triplicate and a quadruplicate was added when one of the results exhibited a discrepancy.

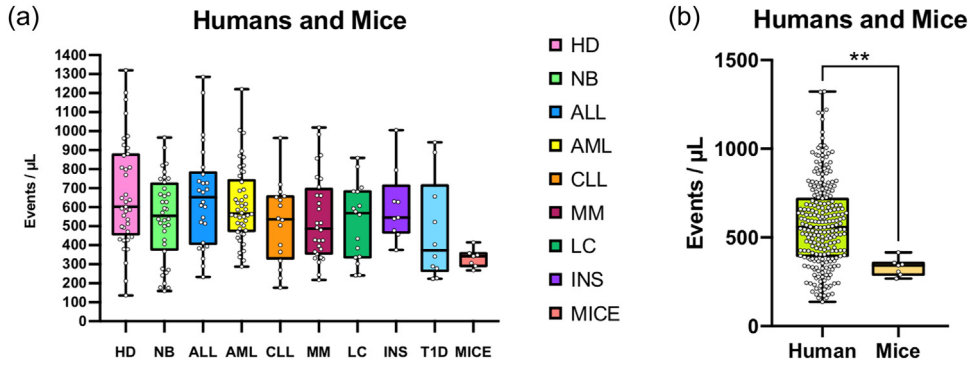


**Fig. 2.** Nanoplastics in healthy donors, newborns, patients with hematological and non-hematological conditions and mice included in this study. Median (m) and range (r) values are shown ($n = 192$). Healthy blood donors (HD, $n = 37$, $m = 614$, $r = 88–1460$), newborns (NB, $n = 36$, $m = 562.1$, $r = 138.9–1038$), patients with acute lymphoblastic leukemia (ALL, $n = 26$, $m = 648.3$, $r = 188–1354$), acute myeloid leukemia (AML, $n = 46$, $m = 577.2$, $r = 238.9–1274$), chronic lymphoblastic leukemia (CLL, $n = 16$, $m = 536.5$, $r = 123.3–1001.1$), multiple myeloma (MM, $n = 28$, $m = 500$, $r = 138.0–1060.8$), non-small cell lung cancer (LC, $n = 16$, $m = 535.2$, $r = 101–992.6$), idiopathic nephrotic syndrome (INS, $n = 9$, $m = 556.4$, $r = 353,6–1077$), type 1 diabetes (T1D, $n = 10$, $m = 368.2$, $r = 140.3–962.9$) subjects and mice ($n = 7$, median=318.1, range=239.5–493.7 events/µL) were included (Fig. 2A). Direct comparison of all human subjects and mice is shown in Fig. 2B, and used to display significant differences in the accumulation of nanoplastics.

at the lowest possible sample rate (12.5 µL/min) and a quadruplicate was added when one of the results exhibited a discrepancy. Limitations of the study rely on the types of plastic that NR is not able to stain, and on the effect of the degradation of organic matter (Supplementary Figure 3). Another limitation is associated with the detection limit of the flow cytometer. In the case of very small particles (i.e. extracellular vesicles), membrane vesicles as small as 70 nm can be detected using a fluorescence threshold. Regarding small particle discrimination (Supplementary Figure 4)-, it has been shown that violet scattered light can be used to identify gold and silver particles between 50 100nm [45]. Regarding polymer size range, little is known based on the differential binding affinity, steric hindrance, and other dye-to molecule interactions. In the case of nucleic acid stains, linear DNA can be sized over a range of 10 to 50 kbp, and this corresponds to analyzing less than 1 pg of DNA [13].

*Plastic accumulation in peripheral blood*

We have prospectively evaluated the accumulation of plastics in blood in a cohort of 196 individuals. With the aim of obtaining reference values for the accumulation of plastics in blood, regular blood donors, newborns, and patients with hematological and non-hematological conditions were included in this study. Our analysis confirms the presence of plastics in all human and mice subjects (Fig. 2). In general, there was high precision among triplicate measurements, with high dispersion between individuals, especially in healthy donors ($n = 37$, $m = 667$ events/µL, $r = 88–1460$ events/µL). The highest levels of NPs were found in ALL ($n = 46$, $m = 648.3$, $r = 188–1354$ events/µL), whereas the lowest were found in T1D ($n = 10$, $m = 368.2$, $r = 140.3–962.9$). Age-based analysis showed that there is a non-significant decline in the accumulation of plastics from 40 to 90 years of age (Fig. 3), suggesting that the accumulation in other tissues, such as adipose tissue, cannot be ruled out. However, these series are still short and incomplete, and more studies will be needed to enlighten how plastics are accumulated with age, as an age-related phenomenon of redistribution and exposure to plastic

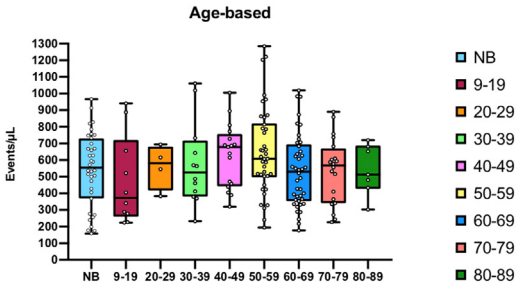


**Fig. 3. Age-based distribution of nanoplastics in peripheral blood.** Age-ranges were partitioned in decades: 9–19 ($n$ = 10, $m$ = 368.2, $r$ = 140.3–962.9), 20–29 ($n$ = 4, $m$ = 570.4, $r$ = 347–786.8), 30–39 ($n$ = 12, $m$ = 548.6, $r$ = 188–1216), 40–49 ($n$ = 17, $m$ = 654.6, $r$ = 293.1–1139), 50–59 ($n$ = 38, $m$ = 614.6, $r$ = 101–1354), 60–69 ($n$ = 41, $m$ = 502.5, $r$ = 123.3–1144), 70–79 ($n$ = 20, $m$ = 557.4, $r$ = 190.4–992.6) and 80–89 ($n$ = 7, $m$ = 546, $r$ = 227–802.4). Newborns ($n$ = 36, $m$ = 562.1, $r$ = 138.9–1038) were characterized by low dispersion measurements.

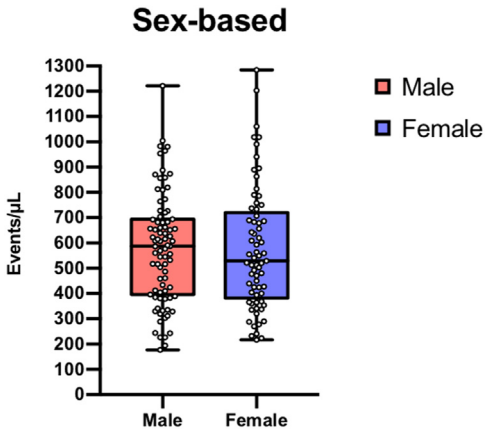


**Fig. 4.** Sex-based distribution of nanoplastics in peripheral blood. A total of $n$ = 150 individuals were included in this study: $n$ = 82 males ($m$ = 577, $r$ = 123,3–1274) and $n$ = 68 females ($m$ = 575.2, $r$ = 101–1354).

pollution. Sex-based analysis (Fig. 4) also provided non-significant differences between men ($n$ = 83, $m$ = 577.04, $r$ = 123.3–1274 events/µL) and women ($n$ = 69, $m$ = 576.2, $r$ = 101–1354 events/µL).

All the subjects involved in this study live in the Metropolitan Area of Barcelona, a highly populated urban agglomeration with problems of air pollution that stands as a potential source of plastic particles for airborne particulate matter inhalation. For this reason, we next decided to study the accumulation of plastics in mice under highly controlled containment conditions. The Comparative Medicine and Bioimage center of Catalonia (CMCiB-IGTP) research infrastructure aims to become a reference center for comparative medicine, surgery, bio-imaging, Biosafety Level 3 (BSL-3) for biomedical studies, and mathematical simulators in Europe, while at the same time developing alternative research methods to the use of animal models. The mouse animal facility at CMCiB allowed us to compare our results in humans with results obtained under specific containment. Peripheral blood analysis of mice maintained at this facility showed the significant low accumulation of plastics when compared with humans ($n$ = 7, median=318.1, range=239.5–493.7 events/µL), suggesting that airborne and other pollutants may play an important role in human plastic accumulation due to the exceptional and rapid accumulation of anthropogenic debris, especially in highly populated areas. If this is true, it will be essential to explore the accumulation of plastics in rural and remote populations to try to monitor how the plastic levels at different places change.

Organic matter degradation was performed in a thermal block at 60 ˚C for a minimum of 10 days. It has been described that mice have on average half the level of triglycerides when compared with human subjects [28]. These mice studies were conducted with the C57 model, one of the most widely used mice strains, with triglyceride values of 88 mg/dl. The experiments that were conducted for peripheral blood nanoplastics analysis used wild-type non-obese diabetic (NOD) mice, that have similar values to healthy human subjects, with triglyceride values of 150 mg/dl [39].This suggests that the difference between events found in human and mouse samples are not due to potential saponification during organic matter degradation. Although other methods can be used for degradation, KOH treatment was preferred because it avoids the use of volatile solvents, such as ethanol, hexane, heptane, or alternately the use of detergents for precipitation and solubilization, that can considerably impact sample volume variation.

Live cell imaging and analysis also helped us to show the inability of phagocytes to engulf or degrade plastic microbeads. In our experiments, we artificially added fluorescent green microspheres for the rapid and accurate detection and quantification of in vitro phagocytosis. As shown in the Supplementary Video I, we have visualized how plastics can impair this fundamental process in

immunity. It has been documented that plastic and plastic-related chemicals, such as phthalates, could disrupt the immune system as well as important metabolic processes, as it has been shown as metabolic disruptors in zebrafish models. Phthalates can interfere with a large number of physiologic and metabolic functions, homeostasis, as well as immune function. Thus, if phagocytes are unable to accomplish phagocytosis with high efficiency, a relevant function against infections will be compromised under the presence of plastics and plastic-related chemicals. Our results suggest that 0.1–0.3% of plastics in blood are MPs, whereas the vast majority is constituted by NPs. Based on different physical interactions such as Brownian motion in cell culture, large particle size can make cell absorption difficult. Understanding the internalization and release of NPs will help to explain how the immune system is impaired and how this may involve nutrient absorption, energy metabolism, cytokine release, inflammatory mechanisms, and disease.

In conclusion, we have demonstrated for the first time the existence of NPs in human peripheral blood detected by flow cytometry. While MPs can be easily seen using microscopy, flow cytometry is a highly sensitive tool to study plastic nanoparticles, with advantages over other techniques, such as Raman spectroscopy, which can resolve generally 1–2 μm particle sizes. Exposure to NPs may lead to accumulation by inhalation and ingestion. Further analyses will be needed to determine association of NP pollution with lifestyle, health and pathology.

## Ethics statements

### *Human samples*

Blood draws were carried out in strict accordance with the principles outlined in the Declaration of Helsinki for human research and after the approval of the Committee on the Ethics of Research of the HUGTIP, Sant Joan de Deu Hospital, and Parc Taulí University Hospital. All subjects enrolled in this study provided their informed consent. For pediatric specimens, signed informed consent was given by the parents of all subjects, and directly by children older than 12 years old. **Mice samples.** The study was approved by the Committee on the Ethics of Animal Experimentation of the HUGTIP and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

M.D.W. and J.A.B work for Thermo Fisher Scientific, which is in the business of selling flow cytometers and flow cytometry reagents. M.W.O. works for Sartorius Stedim North America, Inc., which is in the business of selling live cell analyzers and reagents. M.V-P is co-founder and CSO of Ahead Therapeutics SL, a start-up company dedicated to develop nanoparticle-based immunotherapies for autoimmune diseases. The remaining authors declare no potential conflicts of interest.

## CRediT authorship contribution statement

**Roser Salvia:** Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **Laura G. Rico:** Methodology, Validation, Writing – review & editing. **Jolene A. Bradford:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – review & editing. **Michael D. Ward:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Michael W. Olszowy:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – review & editing. **Cristina Martínez:** Validation, Writing – review & editing. **Álvaro Domingo Madrid-Aris:** Validation, Writing – review & editing. **Joan R. Grífols:** Validation, Writing – review & editing. **Águeda Ancochea:** Validation, Writing – review & editing. **Laia Gomez-Muñoz:** Validation, Writing – review & editing. **Marta Vives-Pi:** Validation, Writing – review & editing. **Eva Martínez-Cáceres:** Validation, Writing – review & editing. **Marco A. Fernández:** Validation, Writing – review & editing. **Marc Sorigue:** Validation, Writing – review & editing. **Jordi Petriz:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

## Data Availability

Data will be made available on request.

## Acknowledgments

This study was supported by Thermo Fisher Scientific and Sartorius Stedim North America, Inc. We thank the CERCA Programme/Generalitat de Catalunya and the Germans Trias i Pujol Research Foundation for institutional support and also acknowledge financial support from the Obra Social la Caixa. LGM is supported by the Health Department of the Catalan Government (Fellowship PERIS PIF-Salut Grant No. SLT017/20/000049). This work has also been supported by positive discussion through the Agency for Management of University and Research Grants (AGAUR, Generalitat de Catalunya), Catalan Research Group (SGR) 2021 SGR 00002. The authors are very grateful to Clara Streiff, Paola Paglia, Sergio Ramon, Lluís Sainz, and Víctor Querol from Thermo Fisher Scientific for all their help in this research field, and to Sara Vergara, Minerva Raya, Silvia Torres, Laura Díaz Cano, Jana Caniego, Ramon

Susanna, Michelle Álvarez and Maria Sangüesa for kindly providing and preparing samples used in this study. Part of this work was done while the corresponding author was principal investigator at the Josep Carreras Leukaemia Research Institute (Spain).

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