



## Research Article

# FACS-based detection of extracellular ASC specks from NLRP3 inflammasomes in inflammatory diseases

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## Abstract

**Introduction:** The apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is crucial for inflammasome assembly and activation of several inflammasomes, including NLRP3. ASC aggregates are detected in human sera post pyroptotic cell death, but their inflammasome origin remains unclear.

**Method:** This study aimed to develop a method to detect ASC aggregates originating from NLRP3 inflammasomes. Initially, human monocytes, macrophages, and THP-1 ASC reporter cells were employed to validate the detection of ASC/NLRP3-positive events through flow cytometry.

**Results:** The presence of ASC/NLRP3 specks was confirmed in cell supernatants from monocytes and macrophages treated with LPS and nigericin or ATP. Flow cytometry analysis identified double-positive specks in patient sera from inflammatory conditions when compared with healthy controls. Elevated ASC/NLRP3 specks were observed in conditions such as cryopyrin-associated periodic syndrome and Schnitzler's syndrome.

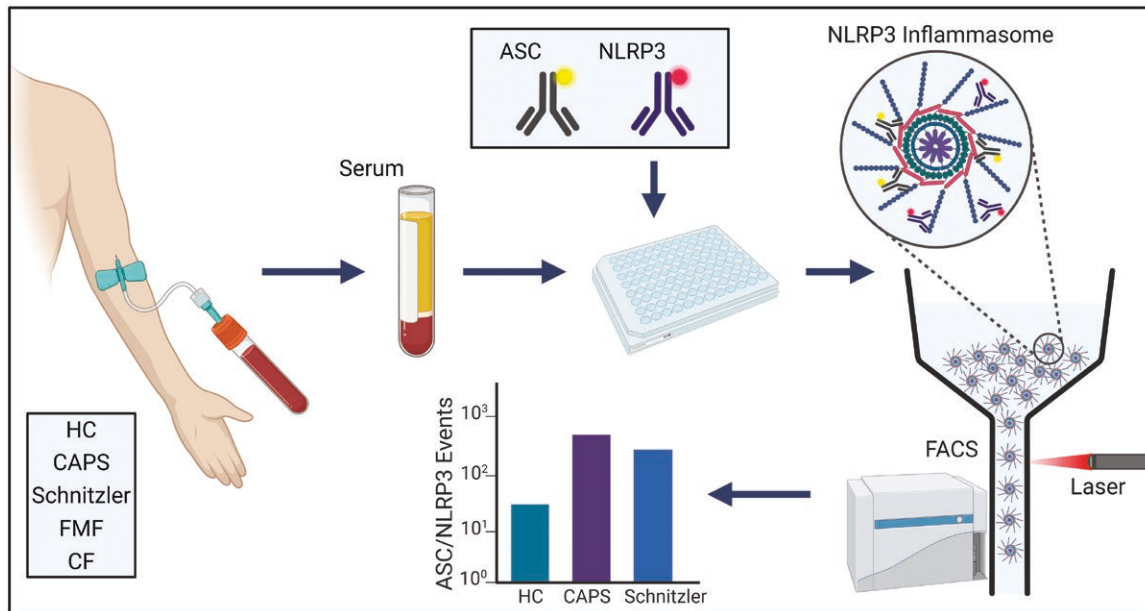
**Conclusion:** We validated fluorescence-activated cell sorting as a reliable method for detecting ASC/NLRP3 specks in human sera, with potential diagnostic and monitoring applications in certain systemic autoinflammatory diseases.

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## Graphical Abstract



**Keywords:** inflammasome, NLRP3, ASC, inflammation, SAID

## Introduction

The NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome is a critical intracellular pattern recognition receptor involved in mediating innate immune responses and release of pro-inflammatory cytokines [1]. Activation of the NLRP3 inflammasome results in the formation of a multimolecular complex containing the sensory component NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and caspase-1 [1, 2]. Central to the inflammasome activation is the re-localization of the ASC protein from being diffusely distributed within the cell, to compression into a dense speck in the cytosol within minutes of the inflammasome activation [2, 3]. This ASC protein aggregate provides a platform for overall inflammasome organization, caspase-1 recruitment, and activation, and ultimately as a support for caspase-1 mediated cleavage of pro-interleukin-1 beta (IL-1 $\beta$ ) and pro-IL-18 into their active forms [4]. The catalytically active ASC specks are released into the extracellular space following cytolitic execution of the pyroptosis [5]. The presence of these extracellular ASC specks has also been detected in the sera of patients with several conditions where the NLRP3 inflammasome activation has been implicated in the disease pathogenesis [5–8].

However, ASC is an adaptor protein, which is also found in other inflammasome complexes including NLRP1, NLRC4, pyrin, AIM2, and NLRP10 [9]. The methodologies used for detecting the ASC specks so far have relied on detecting the ASC protein only, without investigating whether ASC was in complex with another inflammasome component. Therefore, these methods were only partially informative, since they could not reliably determine the inflammasome origin of such specks. Previous studies have shown the potential for extracellular ASC specks to be used as a novel biomarker for both diagnosis and/or disease monitoring [10–12]. The diagnostic utility of the extracellular ASC specks could potentially be improved if the inflammasome receptor at the initiation of

extracellular specks could be accurately identified. To this end, we developed a novel assay which detects ASC/NLRP3 inflammasome events in the sera of patients with inflammatory conditions.

## Materials and methods

### Ethics

Samples were collected from both patients and healthy controls (HCs) using Vacuette tubes (Greiner-Bio-One) containing a serum clot activator gel and EDTA for whole blood (50 ml). Patients' sera were obtained from two distinct sources. Samples were collected from adults with cystic fibrosis (CF) homozygous for F508 $\Delta$ , known to exhibit elevated levels of serum ASC specks. Ethical approval for the study was granted by the Yorkshire and The Humber Research Ethics Committee (17/YH/0084). Sera from HC and patients with various systemic autoinflammatory conditions were sourced from the ImmunAID consortium (IMMUNome project consortium for Autoinflammatory Disorders). This study was approved by the South West—Frenchay Research Ethics Committee (REC Reference: 20/SW/0022). Detailed inclusion and exclusion criteria applied for recruiting HC and patients into the ImmunAID project are provided in the [Supplementary Methods](#).

### Cell lines

THP-1-defASC cells (InvivoGen) or THP-1-ASC-GFP (InvivoGen) were maintained in RPMI with Glutamine (Gibco) supplemented with 10% heat-inactivated FBS (Sigma), and penicillin/streptomycin at 37°C in 5% CO<sub>2</sub> atmosphere. THP-1 cells were used after rested overnight or differentiated with 50-ng/ml phorbol 12-myristate 13-acetate (PMA, P1585, Sigma) in 24-well plates (Starlab) for 3 days, followed by a 2-day rest period by replacing the differentiation medium with complete medium without PMA. Cells were not used beyond passage 11.

## Human monocytes and monocyte-derived macrophages isolation and culture

For isolating primary human monocytes, peripheral blood mononuclear cells were extracted from EDTA blood tubes using a standard density gradient centrifugation method with Lymphoprep and SepMate tubes (StemCell Tech), as described by the manufacturer. CD14<sup>+</sup> monocytes were extracted using magnetic microbeads (130-050-201, MACS Miltenyi). Isolated human monocytes were cultured in RPMI with Glutamine (Gibco) supplemented with 10% human AB serum (H4522, Sigma), and penicillin/streptomycin at 37°C in 5% CO<sub>2</sub> atmosphere. To differentiate monocyte-derived macrophages (MDMs), Roswell Park Memorial Institute Medium (RPMI) was supplemented with 20-ng/ml human GM-CSF (PeproTech) for macrophage differentiation and incubated for 6 days, with fresh media replaced on Day 3 and Day 6. Monocytes were initially seeded at a density of  $0.5 \times 10^6$  cells per well in tissue culture-treated 24-well plates (Starlab).

## Cell treatments and siRNAs

Monocytes were allowed to settle overnight before experimentation, whereas MDMs were stimulated after differentiation on Day 7. Inflammasome activation was achieved by treating the cells with LPS 100 ng/ml (InvivoGen) for 3.5 hours, followed by ATP 5 mM (InvivoGen) or nigericin 20  $\mu$ M (InvivoGen) for the final 30 minutes of stimulation, for a total of 4 hours. MDMs were transfected with OnTarget Plus smart pool siRNAs for NLRP3 (Dharmacon) or Non-targeting Control Pool (Dharmacon) 24 hours before stimulation, refreshing MDM medium before transfection. All siRNAs were used at a final concentration of 30 nM. The transfection mix was prepared as a 10 $\times$  mix in OptiMEM containing the appropriate siRNA and TransIT-X2 transfection reagent (MIR 600 $\times$ , Mirus) in a 1:2 stoichiometry.

## Immunoblotting

For immunoblotting experiments, a total of  $0.5 \times 10^6$  cells were seeded into each well of a 24-well plate. Cells were then subjected to differentiation either as MDMs or THP-1-derived macrophages as described above. Upon differentiation, cells were washed with PBS and subsequently lysed on ice for 5 minutes using 80  $\mu$ l of RIPA buffer supplemented with protease inhibitors (Protease Inhibitor Cocktail set III, EDTA free, Merck) and phosphatase inhibitors (PhosSTOP, Roche). Lysates were then centrifuged at maximum speed for 15 minutes at 4°C, and the cleared lysates were transferred to new tubes. Diluted cleared lysates (1:5) were subjected to the BCA assay (Pierce BCA protein assay kit, 23225, Thermo Scientific) to determine protein concentration. Samples were then diluted in 4 $\times$  Laemmli sample buffer for use in immunoblotting analyses. Subsequently, equal amounts of protein from each sample were run on 4–12% Bis-Tris gels (Novex, Invitrogen) in MES running buffer and transferred onto PVDF membranes using the iBlot transfer system (Invitrogen). Membranes were blocked with 5% BSA in TBS-T for at least 1 hour at room temperature. Incubation with primary antibodies was carried out overnight at 4°C. Membranes were washed 5 times with TBS-T, then probed with their corresponding secondary antibodies (HRP-Conjugated) diluted at 1:5000 in 1% BSA in TBS-T, followed by 5 additional washes with TBS-T. Finally, the membranes were incubated

with SuperSignal West Pico PLUS chemiluminescent substrate (ThermoFisher) for 1 minute, and the signal was captured using a ChemiDoc Imaging System (BioRad). A list of all antibodies used in this study can be found in [Supplementary Table S1](#).

## Immunofluorescence cell microscopy

THP-1 cells were recorded after the corresponding stimulations using the IncuCyte Live Cell Analysis system (Sartorius), with the green channel to detect GFP in the THP-1-ASC-GFP. Supernatants were viewed by Structured Illumination Microscopy on Zeiss Elyra 7 and Zen Black imaging software.

## Flow cytometry

For the detection of ASC specks, or protein specks that contained ASC and NLRP3 in a complex, 1.2  $\mu$ L of PE-conjugated anti-ASC antibody (HASC-71 clone, BioLegend) was used alone, or in combination with 6  $\mu$ L of APC-conjugated anti-NLRP3 (clone 768319, R&D Systems) added to 60  $\mu$ L of cell culture media or serum (1:50 and 1:10 dilutions, respectively). The mixture was incubated in 96-well plates on a shaker for 1 hour. Size gating was carried out with Flow Cytometry Sub-Micron Particle Size beads (ThermoFisher) according to the manufacturer's specifications and was used to threshold out readings below around 0.5, 1, and 2  $\mu$ m. Samples were run and analysed on a CytoFLEX-S (Beckman Coulter), and 30  $\mu$ L of total volume was acquired in slow settings (10  $\mu$ L/minute).

## Cytokine quantification by ELISA

Cytokines were detected by ELISAs IL-1 beta Human Matched Antibody Pair (ThermoFisher), as per the manufacturer's recommendations. In general, ELISA plates were coated with 100- $\mu$ L cytokine capture antibody in PBS overnight at 4°C. The plates were washed three times with PBST (PBS containing 0.5% Tween 20) and the wells were blocked with 300- $\mu$ L assay buffer (0.5% BSA, 0.1% Tween 20 in PBS) by incubating for 1 hour. The plates were washed twice with PBST, and 100  $\mu$ L of sera/culture supernatants, together with appropriate standards, were added to wells in duplicates. Immediately, 50  $\mu$ L of detection antibody was added to all wells and incubated for 2 hours. After incubation, the plates were washed five times with PBST and 100  $\mu$ L of tetramethylbenzidine (TMB) substrate solution (Sigma) was added to all wells and incubated for 30 minutes. Colour development was stopped by adding 100  $\mu$ L of 1.8N H<sub>2</sub>SO<sub>4</sub>, and absorbance was measured at 450 nm with a reference at 620 nm. Note that all incubation steps were done at room temperature with continual shaking at 700 rpm. All data points are an average of duplicate technical replicates for each independent experiment.

## Data handling and statistical analysis

Graphs were generated using Prism 11 (GraphPad) and are shown as the mean of  $N = 3$  experiments, each typically consisting of two technical replicates as indicated. Error bars represent the standard error of the mean. The Kruskal–Wallis test with Dunn's multiple comparison test was performed when comparing non-parametric populations. A two-way ANOVA statistical test with Tukey's multiple comparison post-hoc analysis was performed when calculating variance between

samples ( $P$ -values  $^* \leq 0.05$ ,  $^{**} \leq 0.01$ ,  $^{***} \leq 0.001$ ). Statistical tests used are indicated in the figure legends.

## Results

To investigate the dynamics of ASC speck release into the extracellular fluid and validate detection methodologies, we employed THP-1 cell lines (human leukemia monocytic cell line) genetically modified to be either ASC-deficient (THP-1-defASC) or to express ASC tagged with GFP (THP-1-ASC-GFP), with the latter allowing visualization using fluorescence microscopy. Inflammasome induction and ASC speck formation were achieved using a well-established protocol, involving a longer priming phase with LPS, followed by a shorter NLRP3 induction phase using ATP or nigericin. As expected, only THP-1-ASC-GFP cells formed a single ASC speck post-stimulation with LPS and ATP or nigericin (Fig. 1A), whereas LPS-only stimulation still induced ASC specks to a lesser extent, with most of the ASC diffused in the cytosol (Fig. 1A). To corroborate that ASC specks were secreted into the extracellular space, we imaged the cell-free supernatants of THP-1-ASC-GFP and found ASC specks only in cells stimulated with LPS and ATP or nigericin (Fig. 1B). We analysed and confirmed the presence of ASC, by immunoblotting, in THP-1-ASC-GFP intracellularly and in the supernatants (Fig. 1C). We then used fluorescence-activated cell sorting (FACS) to detect ASC specks in the cell supernatants using a gating restricted to 1  $\mu\text{m}$  in size (Fig. 2A). While the presence of ASC was minimal in all the controls, and in unstimulated cells and in THP-1-defASC cells (Fig. 2B and C), there was a significant increase in ASC events in THP-1-ASC-GFP cells stimulated with LPS and nigericin (Fig. 1D and E). Finally, we detected the presence of mature IL-1 $\beta$ , a marker of inflammasome activation and pyroptotic cell death in the supernatant of THP-1-ASC-GFP stimulated with LPS and nigericin (Fig. 1F).

Next, we investigated the co-detection of ASC and NLRP3 proteins in primary human monocytes and macrophages using FACS. Similarly, in the THP-1 cells, we observed low levels of ASC/NLRP3 specks in all controls and unstimulated monocytes (Figs 3A, and 4A and B). Stimulation with LPS and ATP or nigericin significantly increased the number of ASC/NLRP3 positive events in the 1- and 2- $\mu\text{m}$  gates, but not in the 0.5- $\mu\text{m}$  gate (Fig. 3A and B), confirming the presence of ASC/NLRP3 inflammasome complexes. Additionally, immunoblotting confirmed the presence of ASC and NLRP3 in monocytes (Fig. 3C). To further validate the detection of ASC/NLRP3 events, we used siRNAs targeting NLRP3 or a non-targeting control (CTRL) in human MDMs (Fig. 3D and E). ASC/NLRP3 events were confirmed in the supernatants of MDMs stimulated with LPS and ATP or nigericin (Fig. 3D). These events significantly decreased in MDMs treated with NLRP3 siRNA (Fig. 3D), confirming the specific detection of ASC/NLRP3 events. Additionally, we validated the presence of ASC and NLRP3 by immunoblotting in these cells (Fig. 3E). Finally, to corroborate our data, we also detected ASC/NLRP3 positive events in the supernatants of THP-1 derived macrophages through FACS and confirmed the presence of ASC and NLRP3 by immunoblotting (Fig. 4D and E).

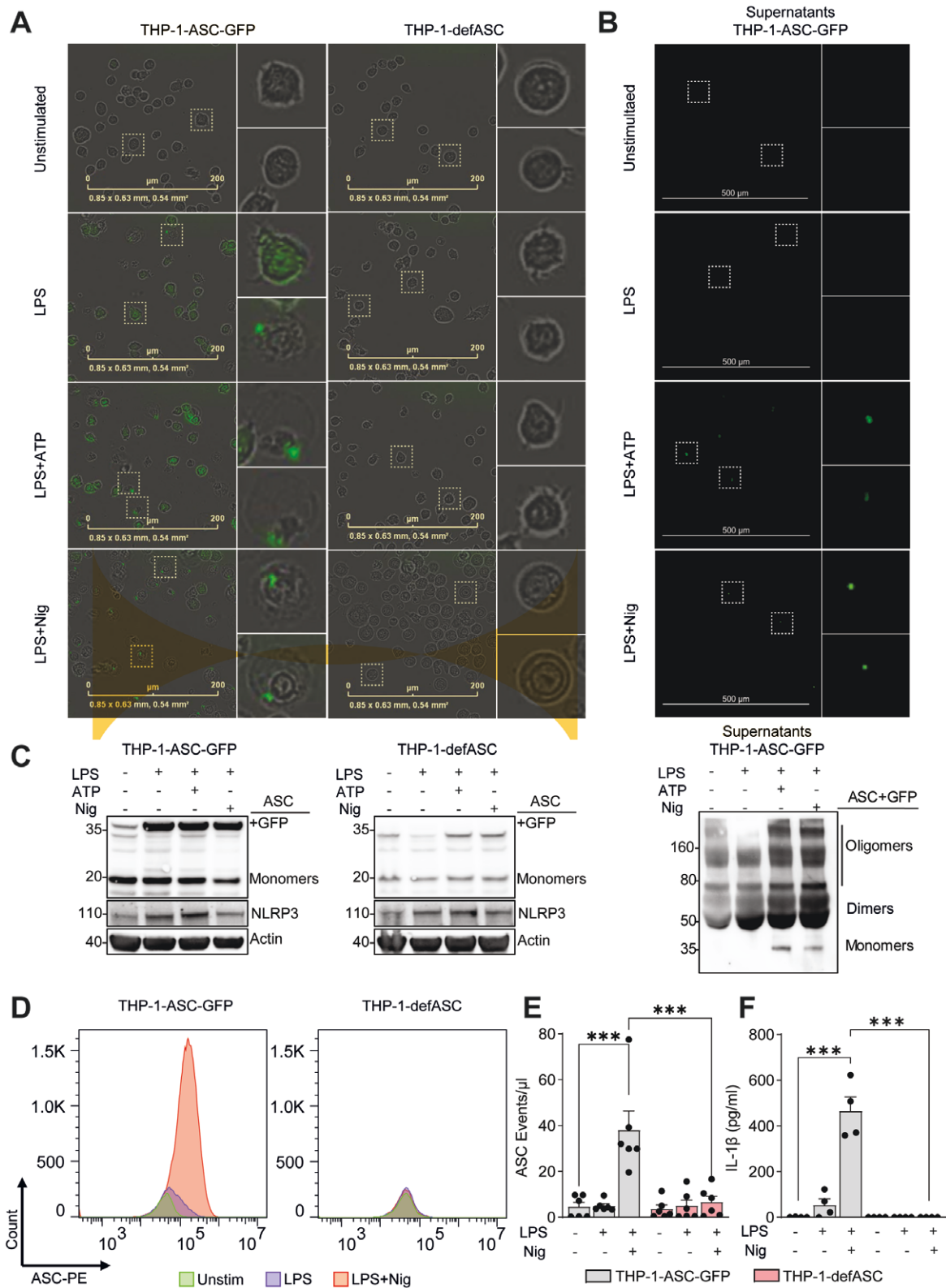
To determine whether ASC specks are detectable under physiological conditions in human sera and if they form complexes with NLRP3, we utilized sera from HCs and patients with inflammatory conditions. Similarly to monocytes

and MDMs, serum from systemic autoinflammatory diseases (SAIDs) showed increased ASC/NLRP3 positive events in the 1- and 2- $\mu\text{m}$  gates (Fig. 5A and B), but not in the 0.5- $\mu\text{m}$  gate (Supplementary Fig. S1A), when compared with HC. Interestingly, ASC/NLRP3 positive events were also significantly increased in sera from patients with CF where events  $>1 \mu\text{m}$  were gated (Supplementary Fig. S1B). We also confirmed the presence of ASC and NLRP3 molecules on human sera, with increased levels of these two proteins in SAID and CF patients (Supplementary Fig. S1C). To assess the diagnostic potential, we tested sera from a large, sex-, and age-matched HC population and compared it to conditions associated with NLRP3 inflammasome activation, such as cryopyrin-associated periodic syndrome (CAPS), which is caused by a gain of function mutations in the NLRP3 leading to constitutional NLRP3 inflammasome activity, and Schnitzler's syndrome, also thought to involve excessive NLRP3 inflammasome activation. Patients with Familial Mediterranean fever (FMF) patients served as controls, given that their condition involves a different inflammasome sensor, pyrin. Our findings revealed that patients with CAPS and Schnitzler's syndrome exhibit significantly elevated levels of ASC/NLRP3 specks compared with HCs (Fig. 5C). Conversely, in FMF patients, ASC/NLRP3 specks were not elevated, aligning with the understanding that the pathogenesis of FMF is more closely associated with a different inflammasome sensor, pyrin (Fig. 5C). Finally, to evaluate the reproducibility of ASC/NLRP3 speck detection using this FACS methodology, we analysed 11 individual samples on two separate occasions, more than 4 weeks apart. Serum sample aliquots were stored at  $-80^\circ\text{C}$  prior to analyses. The results demonstrated highly consistent levels of ASC/NLRP3 specks across the two time points, indicating that this methodology yields reproducible results and that the analyte remained stable despite undergoing one freeze/thaw cycle and extended storage (Fig. 5D).

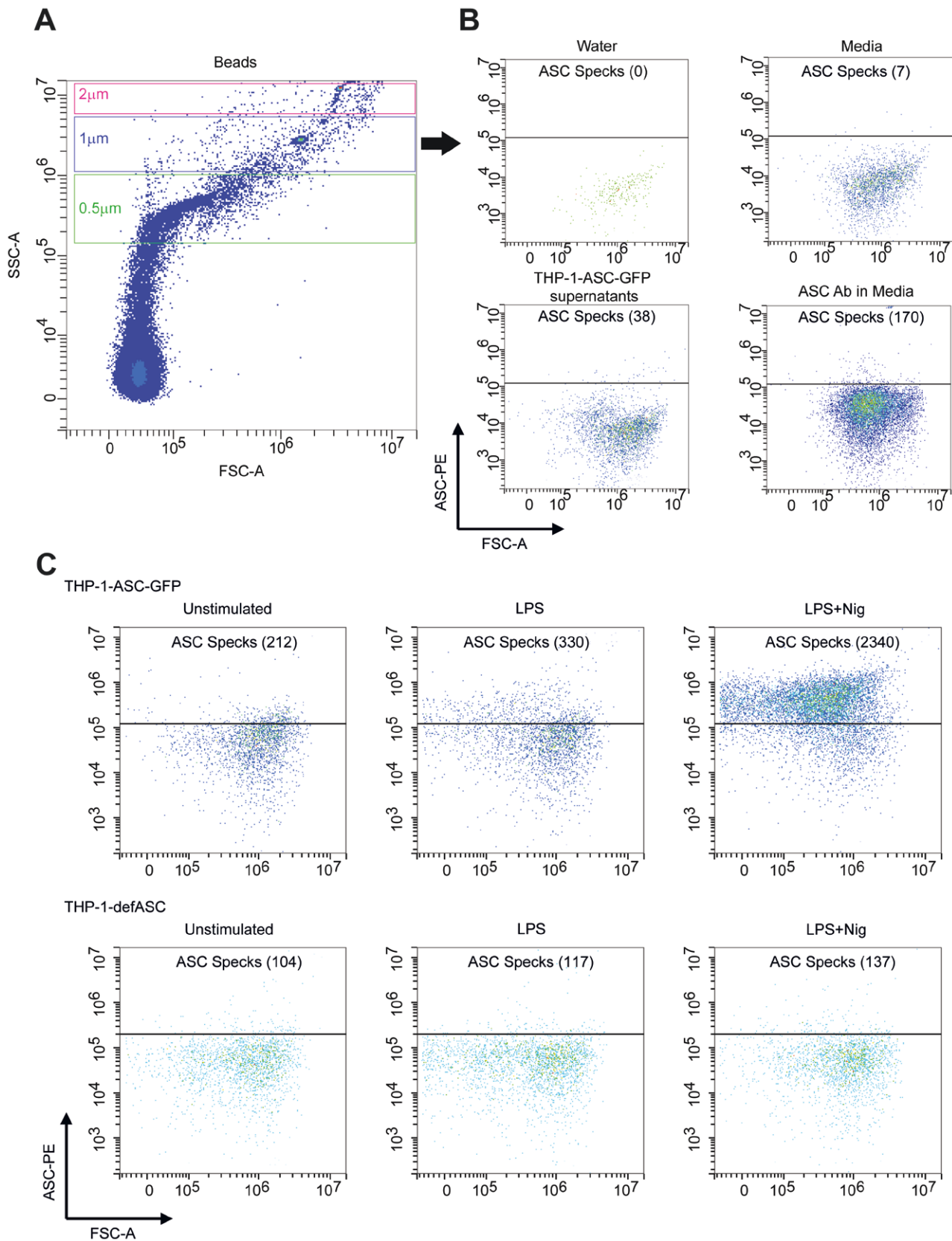
## Discussion

Inflammasomes are critical intercellular platforms that play essential roles in sensing and propagating signals associated with tissue damage and infection [4]. Central to inflammasome activation is the formation of ASC oligomers, which interact with the sensing components defining various inflammasomes (NLRP1, NLRP3, NLRC4, Pyrin, and AIM2), ultimately leading to caspase-1 activation. Caspase-1, in turn, processes several proteins within the cell, including gasdermin D (GSDMD), and the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 [1]. Several studies have suggested that detecting ASC oligomers (specks) in human sera could serve as a novel method to determine inflammasome activation and act as a useful biomarker. Most of these studies implied that ASC specks originate from NLRP3 inflammasome activation, albeit without providing concrete evidence. To address this gap, we refined our ASC speck detection methodology to investigate their origin.

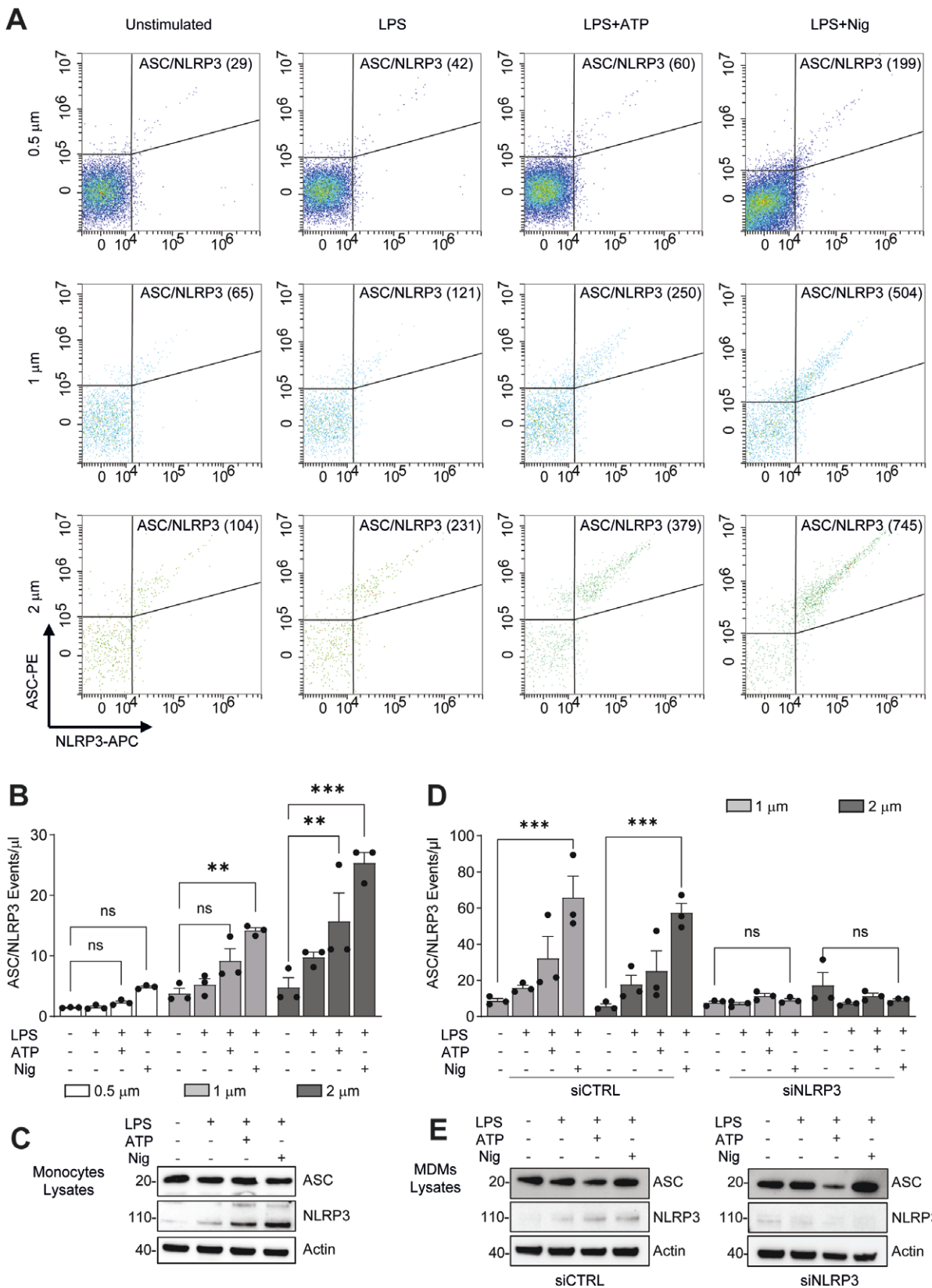
We developed an FACS-based approach that enables the simultaneous detection of ASC specks in serum and the exploration of their association with other inflammasome components, thereby determining their origin. Initially, we validated our methodology using commercially sourced THP-1 cell lines modified for easy detection of ASC monomers and oligomers by attaching a fluorescent GFP tag to ASC,



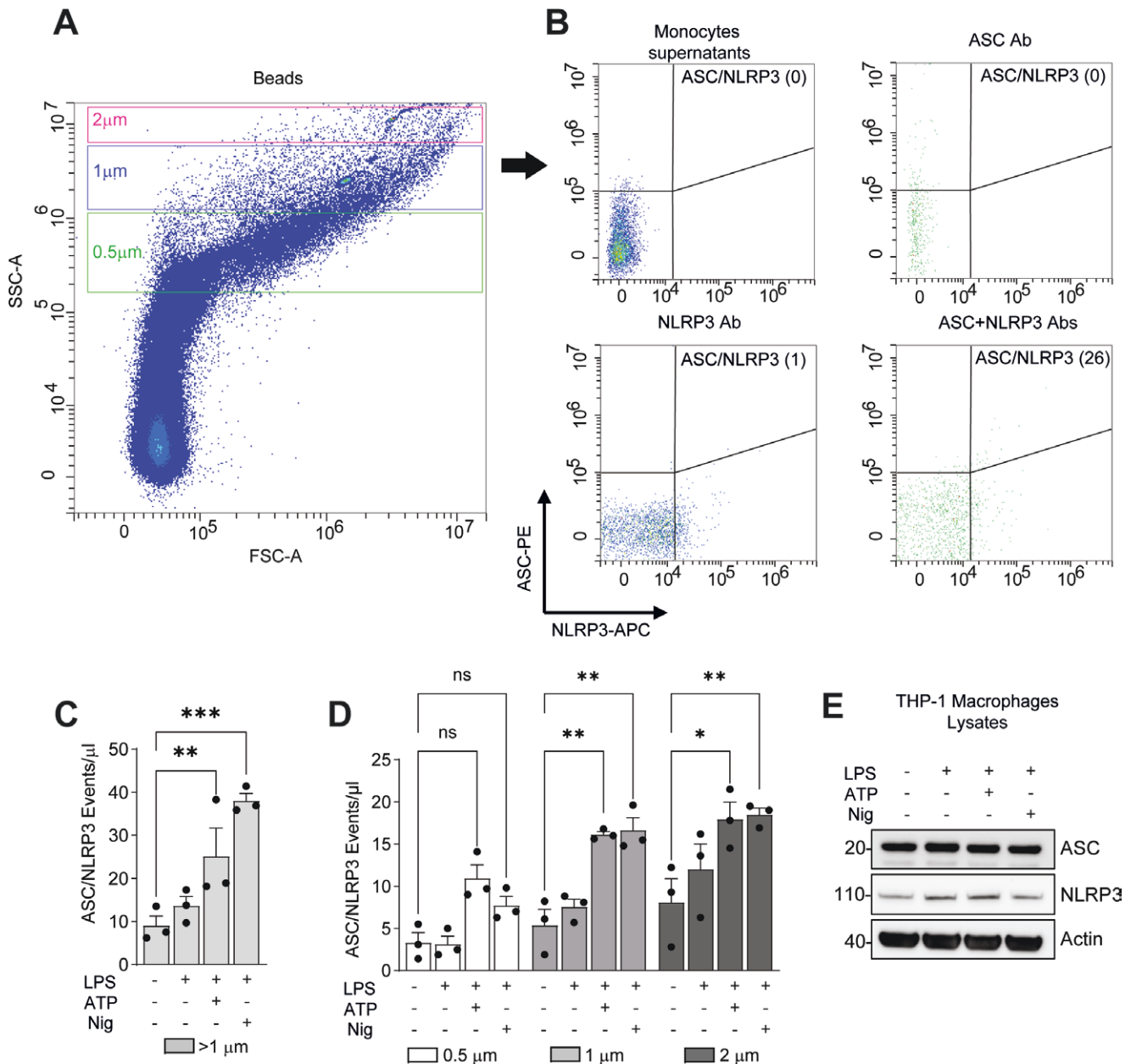
**Figure 1:** Extracellular ASC in THP-1 cells. (A) Detection of ASC specks in THP-1-defASC or THP-1-ASC-GFP by fluorescent microscopy, stimulated with LPS, LPS + ATP, or LPS + nigericin (LPS + Nig) as indicated in the methods. Each dotted square is shown on the right of the panel as magnified.  $n = 3$  independent experiments were carried out. (B) Extracellular ASC was imaged in the CD7; the images presented here are representative of  $n = 3$  independent experiments with 10 images each. (C) Immunoblots of lysates or supernatants of THP-1-defASC or THP-1-ASC-GFP cells treated as indicated. Images are representative of  $n = 3$  independent experiments. (D and E) ASC specks from THP-1-defASC or THP-1-ASC-GFP cells were captured using flow cytometry, with gating around the 1- $\mu$ m bead size, showing the ASC events/ $\mu$ l in 30  $\mu$ l of total volume. (F) ELISA assays were used to detect IL-1 $\beta$  in the supernatants of the cells.  $P$ -values  $\leq 0.05$ ,  $** \leq 0.01$ ,  $*** \leq 0.001$  from two-way ANOVA following adjustment for multiple comparisons.



**Figure 2:** ASC specks validation on THP-1 cells. (A–C) Detection of ASC specks in THP-1-defASC or THP-1-ASC-GFP by FACS, stimulated with LPS, LPS + Nig, or using the indicated control, as described in the methods. Validation of ASC specks using flow cytometry with a gating around the 0.5-, 1-, and 2-µm bead size, showing the ASC events/µl in 30 µl of total volume. The number next to the ASC specks represents the total number of events. A total of six independent experiments were carried out.



**Figure 3:** Secretion of ASC/NLRP3 by macrophages. (A and B) Detection of ASC/NLRP3 specks in the supernatant of primary human monocytes, stimulated as shown in the figure, using flow cytometry with a gating around the 0.5-, 1-, and 2- $\mu\text{m}$  bead size, showing the ASC/NLRP3 events/ $\mu\text{l}$  in 30  $\mu\text{l}$  of total volume. (C) Immunoblots of lysates of primary human monocytes then treated as indicated, immunoblots are representative of  $n = 3$  independent experiments. (D) Detection of ASC/NLRP3 specks in the supernatant of MDMs, as shown in the figure and transfected with siRNA against NLRP3 or non-targeting control (CTRL). (E) Immunoblots of lysates of MDMs treated as indicated. Immunoblots are representative of  $n = 3$  independent experiments.  $P$ -values  $^* \leq 0.05$ ,  $^{**} \leq 0.01$ ,  $^{***} \leq 0.001$  from two-way ANOVA following adjustment for multiple comparisons.



**Figure 4:** ASC/NLRP3 specks on monocytes and THP-1 macrophages. (A and B) Validation of the detection of ASC/NLRP3 specks in human monocytes by FACS; the indicated control is as shown in the figure. Three independent experiments were carried out. Validation of ASC specks using flow cytometry with gating around the 0.5-, 1-, and 2- $\mu\text{m}$  bead size, showing the ASC/NLRP3 events/ $\mu\text{l}$  in 30  $\mu\text{l}$  of total volume. The number next to ASC/NLRP3 specks represents the total number of events. (C) ASC/NLRP3 specks in human monocytes representing the events/ $\mu\text{l}$  in 30  $\mu\text{l}$  of total volume combining the events of 1- and 2- $\mu\text{m}$  gates. (D) Detection of ASC/NLRP3 specks in the supernatant of THP-1 macrophages, stimulated as shown in the figure, using flow cytometry with a gating around the 0.5-, 1-, and 2- $\mu\text{m}$  bead size, showing the ASC/NLRP3 events/ $\mu\text{l}$  in 30  $\mu\text{l}$  of total volume. (E) Immunoblots of lysates of THP-1 macrophages then treated as indicated. Immunoblots are representative of  $n = 3$  independent experiments.  $P$ -values  $^*\leq 0.05$ ,  $^{**}\leq 0.01$ ,  $^{***}\leq 0.001$  from two-way ANOVA following adjustment for multiple comparisons.

alongside ASC-deficient cell lines. Using these models, we induced ASC speck formation with traditional inflammasome stimulants and detected specks both intercellularly and in cell culture supernatants using various techniques, including IF, WB, and FACS. Extending our experiments to human monocytes and MDMs from HCs, we confirmed intercellular ASC specks and their release into cell culture supernatants. Furthermore, we demonstrated the detection of ASC specks in human sera using WB and FACS, and visualized these structures with IF. Through WB and FACS, we also identified sensory NLRP3 components within these ASC specks.

This new FACS methodology to detect ASC specks complexed with NLRP3 could facilitate high-throughput analysis suitable for clinical applications. Employing this approach, we observed significantly elevated levels of ASC/NLRP3 specks in patients with constitutively active NLRP3 inflammasomes (CAPS) compared with HCs.

In summary, we have used several overlapping methods to develop and validate FACS as a high-throughput method for detecting ASC/NLRP3 positive protein specks in human serum. The full diagnostic utility of this assay still needs to be tested in a larger patient cohort and in parallel with





## Acknowledgements

Not applicable.

## Ethical approval

Ethical approval for the study was granted by the Yorkshire and The Humber Research Ethics Committee (17/YH/0084). Sera from HC and patients with various systemic autoinflammatory conditions were sourced from the ImmunAID consortium (IMMUNome project consortium for Autoinflammatory Disorders). This study was approved by the South West—Frenchay Research Ethics Committee (REC Reference: 20/SW/0022). Detailed inclusion and exclusion criteria applied for recruiting HC and patients into the ImmunAID project are provided in the [Supplementary Methods](#).

## Conflict of interest

S.S. received honoraria for participation in advisory board meetings and speaking engagements, travel support, and research grants from SOBI and Novartis. Other authors have no conflicts of interest to declare.

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## Data Availability

The data that support the findings of this study are available from the corresponding author, (S.S.), upon reasonable request.

## Author Contributions

Joanne Topping (Data curation, Formal Analysis, Investigation, Project administration, Writing—review & editing), Samuel Lara-Reyna (Data curation, Formal Analysis, Investigation, Project administration, Writing—original draft, Writing—review & editing), Alice Ibbotson (Data curation, Formal Analysis, Writing—review & editing), Heledd Jarosz-Griffiths (Data curation, Resources, Writing—review & editing), Leon Chang (Formal Analysis, Writing—review & editing), James

Poulter (Formal Analysis, Resources, Writing—review & editing), Daniel Peckham (Resources, Writing—review & editing), Michael McDermott (Conceptualization, Project administration, Resources, Writing—review & editing), and Sinisa Savic (Conceptualization, Formal Analysis, Project administration, Resources, Supervision, Writing—original draft, Writing—review & editing)

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