Peripheral innate immune and bacterial signals relate to clinical heterogeneity in Parkinson's disease

Appendix A

1.Supplementary Methods

1.1 PBMC isolation, Immunocytochemistry and Flow Cytometry

PBMCs were extracted using the standard Ficoll gradient centrifugation method (Ficoll R Paque Plus, GE Healthcare). Cell suspensions obtained following Ficoll gradient separation were centrifuged, and cell pellets were blocked with fluorescence activated cell sorting (FACS) buffer with 2% mouse serum (Sigma) per 0.5–1 × 10^6 cells. Following blocking for 30min, the PBMCs were stained with a panel of conjugated antibodies detailed in Table A.1 and incubated at 4°C for 30min. Following incubation, the PBMCs were washed, fixed with 2% paraformaldehyde (PFA) and then re-suspended in FACS buffer for flow cytometry. Flow cytometry was performed using the BD LSR Fortessa machine with BD FACS Diva software.

Monocytes were gated as described in the literature (Ziegler-Heitbrock and Hofer, 2013) (Givan A.L., Flow cytometry: first principles, 2001) and a minimum number of 10,000 monocyte events were collected per sample. PBMCs from healthy controls, labeled with single conjugated antibodies, were used to determine the appropriate compensation for spectral overlap of fluorophores. Flow cytometry data was analyzed using Flow Jo software, version 10. The percentage of marker positive cells and marker expression levels were determined with reference to isotype control samples (Median Fluorescence Intensity (MFI) Test/Isotype ratio).

The PBMC extractions and staining were performed throughout at 4°C, limiting cell death. During preparatory work for this study, a Live-Dead differentiation stain (LIVE/DEAD™ Fixable Violet Dead Cell Stain, ThermoFisher) was used to assess the percentage of dead cells (Figure A.1). The majority of monocytes (~99.8%) were negative for the Live-Dead stain and positive numbers were not considered to significantly influence the final analysis. Therefore, the Live-Dead stain was not used routinely in this study.

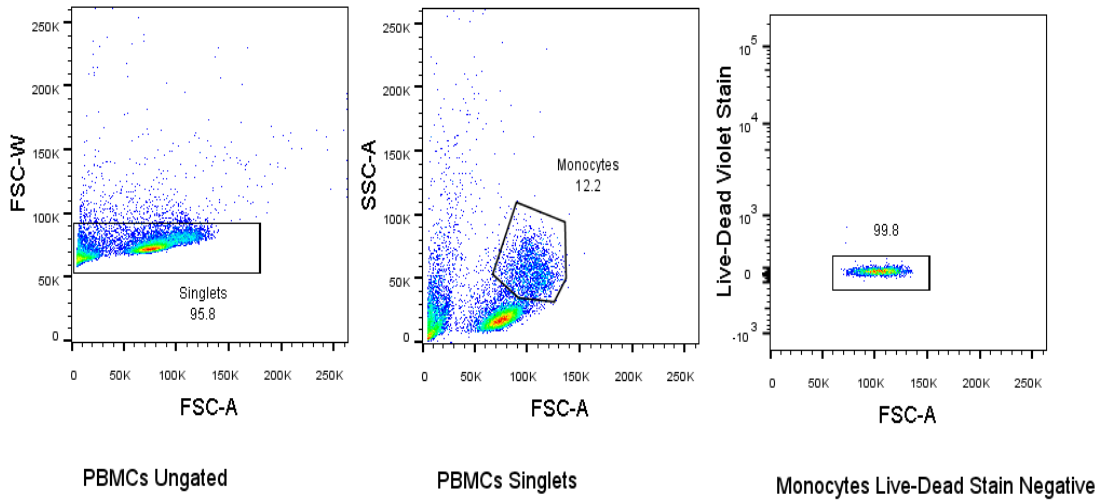

Figure A.1- Live-Dead stain – *Flow cytometry plots demonstrating the use of the Live-Dead stain ex-vivo for estimation of the percentage of dead cells.*

Flow cytometry analysis was performed on total monocytes using a standard method based on a tight gate using the FSC-SSC plot (Figure 1A) (Givan AL. Flow cytometry : first principles. Wiley-Liss 2001)(Tadema et al., 2011), due to the availability of more complete data (problems with the CD14-CD16 staining in a few samples). While this gate may also include a small proportion of related innate immune cells such as dendritic cells, available data indicated that >91% of the FSC-SSC gated monocytes were also monocytes based on CD14-CD16 plot gating.

Additional analysis was performed on Classical (CD14high/CD16negative), Intermediate (CD14high/CD16positive) and Non-Classical (CD14low/CD16high) monocytes. However, the numbers of captured Intermediate and Non-Classical monocytes per sample were considered too low for sufficiently robust analysis and conclusions in this study.

Antibody (anti-Human)	**Isotype Control**	**Volume used (µl)**
CD14 – APC-H7 (MφP9, mouse mAb, BD Biosciences)	Mouse IgG2b κ (27-35, mouse mAb, BD Biosciences)	2
CD16 – PerCP-Cy5.5 (3G8, mouse mAb, BioLegend)	Mouse IgG 1 κ (MOPC-21, mouse mAb, BioLegend)	1
HLA-DR – BV605 (L243, mouse mAb, BioLegend)	Mouse IgG 2a κ (MOPC-173, mouse mAb, BioLegend)	5
TREM2 –APC (#237920, rat mAb, R and D Systems)	Rat IgG 2B (#141945, rat mAb, R and D Systems)	10
TLR2 – PE (TL2.1, mouse mAb, Biolegend)	Mouse IgG 2a κ (MOPC-173, mouse mAb, BioLegend)	5
TLR4 – BV421 (X40, mouse mAb, Biolegend)	Mouse IgG 2a κ (X40, mouse mAb, BD)	5

Table A.1- Antibodies used for immunocytochemistry and flow cytometry.

CD – Cluster of Differentiation; HLA-DR (Human Leukocyte Antigen-DR), TREM2 (Triggering Receptor Expressed on Myeloid cells 2); TLR (Toll like Receptor).

1.2 Serum sample processing and assays

Blood samples taken for serum collection were left to clot for 15 minutes. Samples were centrifuged at 2000rpm for 15 minutes at room temperature. The separated serum was stored in 200-400 µl aliquots and frozen at -80°C.

Mesoscale Discovery (MSD) platform electrochemiluminescence assays (https://www.mesoscale.com).

MSD assay general protocol

Samples were analysed according to the manufacturer's instructions. Briefly, serum samples, stored at -80ºC, were thawed to room temperature. The samples were diluted as specified in the appropriate buffer and loaded in duplicate onto the MSD plates at the specified volume per well. A serial dilution series of manufacturer provided standard samples were also loaded in duplicate. The plate was incubated at room temperature on a shaker for the appropriate time period and washed three times using Phosphate Buffered Saline (PBS) with 0.05% Tween (wash buffer). The specified volume of detection antibody was added at the appropriate concentration and the plate was incubated on the shaker for a further time period. Following removal of the detection antibody, the plate was washed three times and 150µl of 1X or 2X Read buffer was added to each well. Readings were obtained using the MSD SECTOR Imager. Data was exported and analysed using the MSD Discovery Workbench software.

MSD V-Plex Pro-inflammatory panel 1 assay

Samples were analysed for Interferon (IFN)-γ, Interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and Tumour Necrosis Factor (TNF)-α in a multiplexed 10-spot 96 well

plate. Samples were diluted 1:2 and used at 50ul per well. 25µl of the detection antibody and 2X Read buffer were used. Both the initial and second incubation times were 2 hours.

MSD V-Plex Human CRP assay

Samples were analysed for C-Reactive protein (CRP) in a single spot 96 well plate. Samples were diluted 1:1000 and used at 25ul per well. 25µl of the detection antibody and 1X Read buffer were used. The initial incubation period was 2 hours, while the second incubation period was 1 hour.

MSD Human Alpha-Synuclein Assay

Samples were analysed for alpha-synuclein in a single spot 96 well plate. Samples were diluted 1:10 and used at 25ul per well. 25µl of the detection antibody was added together with the samples and the plate was incubated only once for 2 hours prior to washing. 2X Read buffer were used.

Serum Endotoxin – Limulus Amoebocyte Lysate (LAL) Assay

The assay was carried out using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) (40 samples) and the LAL Chromogenic Endpoint Assay (Hycult Biotech) (36 samples), due to supplier shortages during the time of performing the assays. Samples from patient and control pairs were analysed in the same plate using the same kit. The results of samples from both batches covered similar ranges (Thermo Scientific kit – 0.46 – 6.32 EU/ml; Hycult Biotech kit – 0.46 – 5.00 EU/ml).

Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific)

The assay was performed in duplicate according to the manufacturer's instructions. Briefly, following pre-incubation of the microplate at 37ºC, 50µL of each standard or sample (diluted 1:50) was inserted into the appropriate microplate well. The plate was covered and incubated for 5 minutes at 37ºC. 50µL of LAL was added to each well following which the plate was incubated at 37ºC for 10 minutes. 100µL of substrate solution was added to each well, followed by incubation at 37ºC for 6 minutes. 50µL of Stop Reagent (25% acetic acid was added and the absorbance at 405-410nm was measured using the FLUOstar Omega microplate reader (BMG LABTECH). The average absorbance of the blank replicates was subtracted from the average absorbance of all individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected absorbance for each standard versus its concentration in EU/mL and this was used to determine the endotoxin concentration of each unknown sample.

LAL Chromogenic Endpoint Assay (Hycult Biotech)

The assay was performed in duplicate according to the manufacturer's instructions. Briefly, 50µl of sample (diluted 1:50) or standard was added to the assigned wells. 50µl/well of reconstituted LAL reagent was added and the plate was covered and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50µl 1x stop solution and the absorbance at 405 nm was measured using the FLUOstar Omega microplate reader (BMG LABTECH). A standard curve was prepared by plotting the average blank-corrected absorbance for each standard versus its concentration in EU/mL and this was used to determine the endotoxin concentration of each unknown sample.

Serum Caspase 1 ELISA

Samples were analysed using the Human Caspase 1 Quantikine ELISA kit (R and D Systems). The assay was carried out using the manufacturer's instructions and samples were analysed in duplicate. Samples from patient and control pairs were analysed on the same plate. Briefly, 50µL of Assay Diluent was added to each well, followed by 100µL of Standard or sample applied in duplicate. The plate was incubated for 1.5 hours at room temperature on a shaker, followed by aspiration and three washes with ~400µl of wash buffer per well. 100µL of Caspase-1 Antiserum was added to each well and incubated for 30 minutes at room temperature. The aspiration/wash process was repeated and 100µL of Human Caspase-1 Conjugate was added to each well, followed by a further incubation of 30 minutes. The aspiration/wash process was repeated and 200µL of Substrate Solution was added to each well, followed by 20 minutes incubation protected from light. 50µL of Stop Solution was then added and the optical density of each well was determined within 30 minutes, using the FLUOstar Omega microplate reader (BMG LABTECH) set to 450 nm. Readings at 540 nm or 570 nm were subtracted from the readings at 450 nm to correct for optical imperfections in the plate. In calculating the results, the average zero standard optical density was subtracted from all readings. A standard curve was plotted and used to calculate the caspase-1 concentrations for the samples.

Serum soluble TREM2 ELISA

Samples were analysed using the Cloud-Clone Corp. ELISA kit for TREM2. The assay was carried out using the manufacturer's instructions and samples were analysed in duplicate. Samples from patient and control pairs were analysed on the same plate. Samples were diluted 1:2 with PBS based on the manufacturer's guidance and sample trials.

100µL of standard or sample was added to each well, followed by incubation for 1 hour at 37°C. This was aspirated, followed by the addition of 100µL of Detection Reagent A and further incubation for 1 hour at 37°C. The wells were aspirated and washed three times, followed by the addition of 100µL of Detection Reagent B and a further incubation for 30 minutes at 37°C. The wells were then aspirated and washed five times, followed by the addition of 90µL of Substrate Solution and incubation for 10-20 minutes at 37°C protected from light. 50µL of Stop Solution was then added and the absorbance was read immediately using the FLUOstar Omega microplate reader (BMG LABTECH) at 450nm.

In calculating the results, the average zero standard optical density was subtracted from all readings. A standard curve was plotted and used to calculate the soluble TREM2 concentration in the samples.

Serum soluble CD14 ELISA

Samples were analysed using the Human soluble CD14 Quantikine ELISA kit (R and D systems). The assay was carried out using the manufacturer's instructions and samples were analysed in duplicate. Samples from patient and control pairs were analysed on the same plate. Samples were diluted 1:800 based on the manufacturer's guidance and sample trials. Briefly, 100µL of Assay Diluent was added to each well, followed by 100µL of standard or sample per well in duplicate. The plate was sealed and incubated for 3 hours at room temperature. Following this all wells were aspirated and washed 4 times using ~400µl of Wash Buffer per well. 200µL of Human CD14 Conjugate was added to each well, followed by a further incubation for 1 hour at room temperature. The plate was then washed 4 times as above and 200µL of Substrate Solution was added to each well, followed by incubation for 30 minutes at room temperature, protected from light. 50µL of Stop Solution was added to each well and the

optical density of each well was determined within 30 minutes, using the FLUOstar Omega microplate reader (BMG LABTECH) set to 450 nm.

Readings at 540 nm or 570 nm were subtracted from the readings at 450 nm to correct for optical imperfections in the plate. In calculating the results, the average zero standard optical density was subtracted from all readings. A standard curve was plotted and used to calculate the soluble CD14 concentration in the samples.

1.3 Monocyte separation

CD14+ cells were separated from PBMCs using MACS® magnetic CD14+ beads (Miltenyi Biotec) and "LS" columns, according to the manufacturer's instructions. Both manual and automatic methods of MACS® magnetic bead cell separation (Miltenyi Biotec) were used due to limited availability of equipment. Both methods produced similar results in terms of cell purity and yield. The majority of the samples were separated manually, and all patient and control pairs were separated using the same method.

Manual separation

PBMCs were re-suspended in cooled 'MACS' buffer (1XPBS, 5mg/ml BSA, 2mM EDTA) at a concentration of 80µl/10^7cells. The CD14 magnetic bead suspension (Miltenyi Biotec) was added to the cells at a concentration of 20µl per 10^7 cells and incubated at 4ºC for 30 minutes. The cells were washed and re-suspended in ~1ml of MACS buffer. This was added to a pre-rinsed 'LS' separation column which was placed within the MidiMACS™ magnet separator (Miltenyi Biotec). Once the solution had run through, the column was washed three times with 3ml cool MACS buffer. Finally, the column was removed from the magnet, allowing the magnetic bead labelled cells (CD14+) in the column to be eluted with 5ml of MACS buffer into a 15ml Falcon tube.

Separation using the autoMACS® Pro Separator machine

The autoMACS machine (Miltenyi Biotec) was programmed for CD14 positive selection and the CD14+ beads and the sample and collection tubes were placed appropriately for automatic labelling and separation. The programme was run, and the negative and positive fractions of cells were collected in 15ml Falcon tubes.

1.4 Monocyte uptake assays

Fluorescent alpha-synuclein endocytosis assays

Titration and time course experiments were performed prior to the start of the study to optimise the concentrations and end time points for the assays.

Uptake assay protocol

CD14+ cells were centrifuged at 350g for 5 minutes and re-suspended in clear RPMI (Roswell Park Memorial Institute culture medium) (Life Technologies) + 10% FCS (Foetal Calf Serum) (Sigma) (200µl per 0.5 x 10^6 monocytes). The cells were placed in 96 well plates at a concentration of 0.5 x 10^6 monocytes in 200ul per well and equilibrated in the incubator (37ºC, 5% CO2) (Test plates) or the fridge (4ºC plates) for 45minutes.

Following this, recombinant human alpha-synuclein (1-140) HiLyte™ Fluor 488 (Anaspec) (2µl of 1µg/µl solution to give a concentration of 10 ng/µl or 10,000ng/ml) was added and mixed into the appropriate wells. The Test plates were placed in the incubator (37ºC, 5% CO2) to simulate in vivo conditions, while the 4ºC plates were placed in the fridge, as monocyte phagocytosis and endocytosis should be inhibited at 4ºC and were used as a reference to account for any non-specific adherence of the proteins to the cells.

The concentration of alpha-synuclein was based on titration experiments, which used concentrations of 100, 1000 and 10,000 ng/ml. Minimising the time period of uptake, while enabling adequate quantification using flow cytometry, required the use of a high concentration of 10,000ng/ml of alpha-synuclein over 90 minutes, which may be representative of localised rises in alpha-synuclein potentially occurring around aggregates, or following cellular disruptions such as the death or lysis of neurones.

At the end of the period of alpha-synuclein incubation (90 minutes), all plates were placed on ice and ~70-90ul of ice cold PBS was added to the well, followed by centrifugation at 350g for 5 min, discarding of the supernatant and re-suspension in 180µl of ice cold PBS. The cells were then transferred to a 96 well V bottomed plate on ice. This was centrifuged at 350g for 5 min and the supernatant was discarded. The remaining cells were washed in FACS buffer and re-suspended in 100µl of 2% PFA to fix the cells. Following incubation for 15-20 minutes, the samples were washed twice, re-suspended in FACS buffer and analysed using flow cytometry.

Flow cytometry

Flow cytometry was performed on the endocytosis assay samples using the BD LSR Fortessa machine and all monocyte events were recorded. Data was processed using Flow Jo software, version 10. The total monocyte percentage positive uptake and MFI ratio values for uptake were calculated based on the 4ºC samples and as described in the Assay Optimisation section below.

Uptake assays in serum medium

The uptake assays described above were also performed simultaneously with the participant's autologous serum, instead of RPMI and 10% FCS. Serum was isolated from blood as described in the Methods section. The extracted serum was kept at 4ºC until use on the same day.

Microscopy

A subset of the monocyte post uptake samples was used for microscopy. A proportion of the fixed monocyte sample was incubated with the nuclear staining Hoechst dye (Thermofisher Scientific) (1:1000) for 30 minutes and then washed with PBS. The cells were re-suspended in PBS and smeared onto a glass slide and air dried with protection from light. A glass cover slip was applied onto the slide with FluorSave™ reagent solution. Fluorescent microscopy was performed using the Leica DM6000 B microscope, using the blue channel (A4 filter cube) for Hoechst and the green channel (L5 filter cube) for fluorescent alpha-synuclein-488.

1.5 Monocyte alpha-synuclein secretion assays

Monocyte culture

Separated monocytes were resuspended in RPMI and 10% FCS at a concentration of $1x10^6$ cells per ml. $1x10^6$ cells were added per well into a 24 well culture plate with and without lipopolysaccharide (LPS)(Sigma) (1ng/ml). The cells were cultured for 24 hours at 37ºC and 5% CO_2. At 24 hours, the contents of each well were removed and centrifuged at 350g for 5 minutes. The supernatant was collected, aliquoted into cryovials and stored at -80ºC.

1.6 Monocyte Lysates

Lysis protocol

Available separated monocytes were homogenized in a solution containing Tris-buffered saline (TBS), 1% Triton X100 (Sigma T8787), Complete protease inhibitor (Roche 04693132001), extraction buffer (AbCam ab193970), and enhancer (AbCam ab193971) on ice for 30 minutes. The resulting solution was centrifuged at 15,000rcf at 4ºC for 20 minutes. The supernatant was removed and assayed for protein concentration.

Bicinchoninic acid (BCA) assay

Protein concentration of the lysates was determined according to the manufacturer's instructions (Pierce 23225). Briefly, 50 parts of Reagent A was mixed with 1 part of Reagent B and appropriate volumes of the mixture were mixed with a protein sample. The resulting solution was incubated at 37ºC for 30 minutes and absorption was measured using the Nanodrop reader. The protein concentration was calculated using a standard curve of protein concentration versus absorbance for the BCA assay.

Western Blots

Western blot protocol

Monocyte lysate samples were mixed with 0.1M dithiothreitol (DTT) and 4X lithium dodecyl sulphate (LDS) at the appropriate volumes and boiled at 70ºC for 10 minutes followed by centrifugation at 13,000rpm for 5 minutes to remove air bubbles. The samples were then loaded onto the wells of a NuPAGE 10% BIS-TRIS gel (Thermofisher) in 1X 3-(N-morpholino) propanesulphonic acid (MOPS) running buffer (Thermofisher NP0001), together with the protein ladder. The gel was run at 85V for 2 hours, 10 minutes and then transferred onto the membrane in 1X Transfer buffer (Thermofisher NP0006)+ 20% methanol.

The membrane was blocked with 5% bovine serum albumin (BSA) or 5% milk in TBS+Tween 20 (Sigma P9416) (TBST) for 1 hour at room temperature, on a shaker. The primary antibody (alpha-synuclein Syn42 (610786, Mouse mAb, BD Bioscience) was added in blocking buffer at the appropriate dilution (alpha-synuclein 1 in 2000) and left overnight at 4ºC on a shaker. On the following day, the primary antibody was washed off 3 x 10 minutes in TBST. The horseradish peroxidase (HRP) conjugated secondary antibody (Sigma A9044) was added in blocking buffer (1 in 5000), followed by incubation for 1 hour at room temperature on a shaker and thorough washing in TBST.

The blot was visualised using the SuperSignal^{TM} kit (Thermofisher). The membrane was placed on top of sealer plastic and a mixture of peroxidase and enhancer was added at a 1:1 ratio on to its surface. This was incubated in the dark for 5 minutes, followed by development of the signal using the UVI TECH Alliance machine.

Following the first staining and visualisation, the membrane was stripped by incubation in Stripping buffer (15g/L glycine (Sigma G8898), 1g/L sodium dodecyl sulphate (Sigma 75746) and 1% v/v Tween 20) for two 30-minute periods, followed by washing in TBST. The membrane was then stained with the caspase-1 antibody (D7F10, Rabbit mAb, Cell Signalling Technology 1/1000; secondary Sigma AP307P, 1/5000) following the same procedure. Subsequently the membrane was stripped again and stained for β-actin (sc-47778, Mouse mAB, Santa Cruz) as the loading control.

The intensity of the appropriate visible bands for both alpha-synuclein and caspase-1 were analysed using Image J software and normalised to β-actin.

2. Assay Optimisation

2.1 Monocyte fluorescent alpha-synuclein uptake assay

The fluorescent alpha-synuclein used (alpha-synuclein (1-140) HiLyte^{TM} Fluor 488) was found to contain 0.75 EU/µl of endotoxin contamination (Lonza Bioscience) due to the manufacturing process. Previous studies have shown that this fluorescence tagged alpha-synuclein product has similar aggregation properties to untagged alpha-synuclein (Anderson and Webb, 2011), but it is possible that the presence of endotoxin may influence certain processes such as fibril formation (Kim et al., 2016). However, the same alpha-synuclein product and conditions were used for all patient and control assays in this study.

Preparatory assays were performed using young control monocytes to optimise the concentration of fluorescent alpha-synuclein used and the time course of the experiment (Figures A.2, A.3, A.4). Fluorescent alpha-synuclein concentrations of 100ng/ml, 1000ng/ml and 10,000ng/ml were trialled at time points of 15, 30, 60 and 90 minutes. As the intention was to minimise the time period of the uptake assays, time points beyond 90 minutes were not tested. A concentration of 10,000ng/ml at the 90-minute time point produced sufficiently distinguishable levels of uptake to use in the final assays.

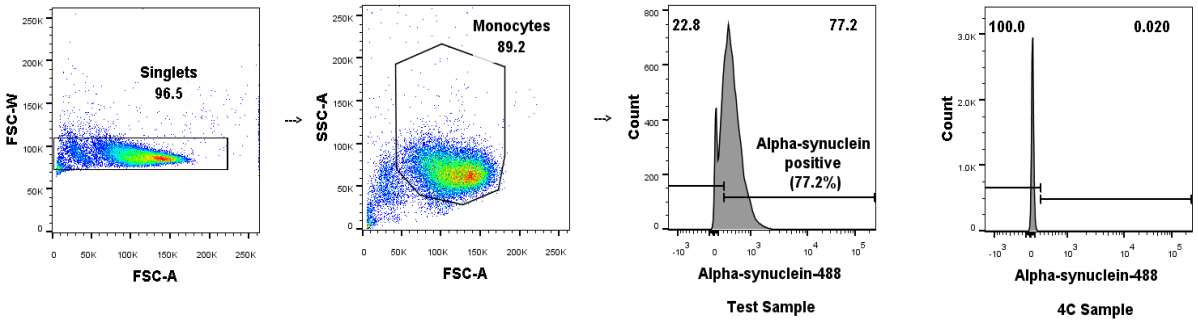

Figure A.2 - Flow cytometry gating strategy for monocyte alpha-synuclein-HiLyte-Fluor-488 uptake. *Monocyte gate extended upwards to include positive monocytes, which may have increased side scatter. Dividing gate on histogram based on position of 4ºC sample. FSC-A=Forward scatter –Area; FSC-W = Forward scatter-width; SSC-A= Side scatter-Area*

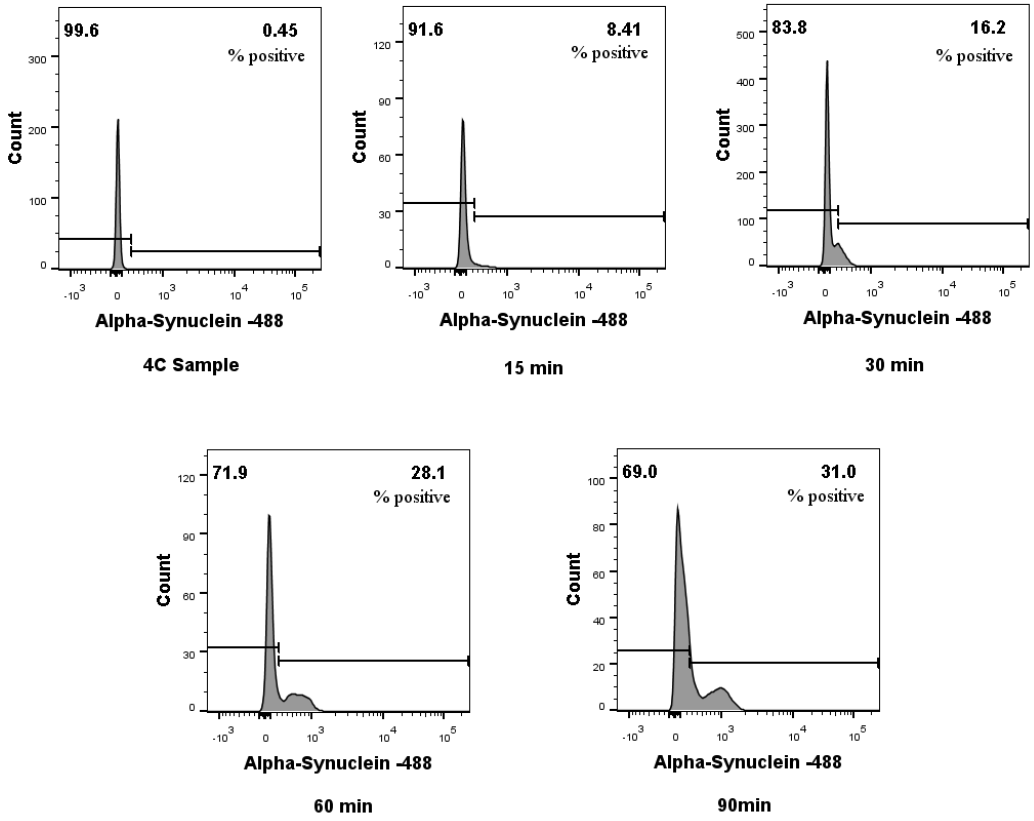

Figure A.3 – Time course of fluorescent alpha-synuclein-488 uptake. *Alpha-synuclein concentration used - 10ng/µl (10,000ng/ml). Subsequent assays were performed at 90 minutes throughout the study. % positive uptake is based on 4ºC sample.*

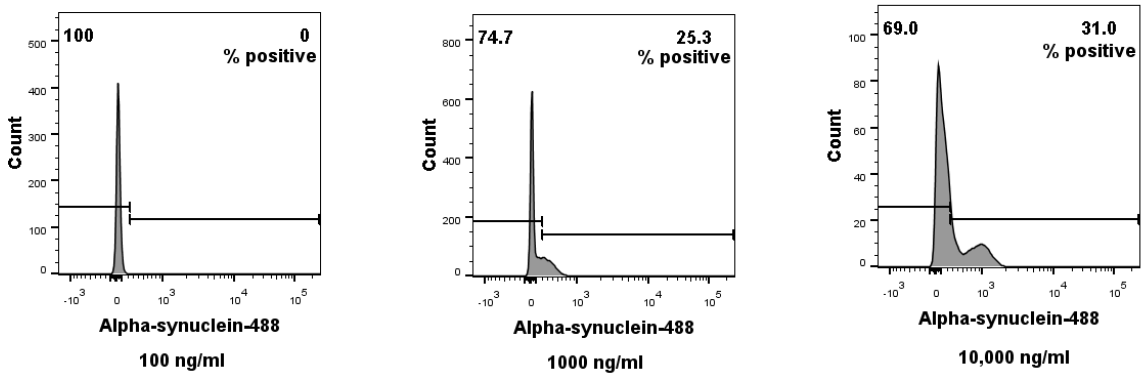

Figure A.4 - Titration of alpha-synuclein concentration. *Examples of uptake at 90-minute time point at concentrations of 100ng/ml, 1000ng/ml and 10,000ng/ml. % positive uptake is based on 4ºC sample.*

The outcomes measured in these fluorescent alpha-synuclein assays included the percentage of monocytes which had taken up alpha-synuclein compared to the 4ºC sample (Test sample % positive - 4ºC sample % positive) and the MFI ratio of total monocytes (Test sample total monocyte MFI/ 4ºC sample MFI).

3. Supplementary Results

3.1 Monocyte subtypes analysis

The Parkinson's disease (PD)-Control differences for each marker in each subtype (Figures A.5, A.6, A.7) showed overall similar trends to that seen in total monocytes (Figure 2). However, the numbers of captured Intermediate (CD14high/CD16positive) and Non-Classical (CD14low/CD16high) monocytes per individual were considered too low for sufficiently robust analysis and conclusions to be made in this study.

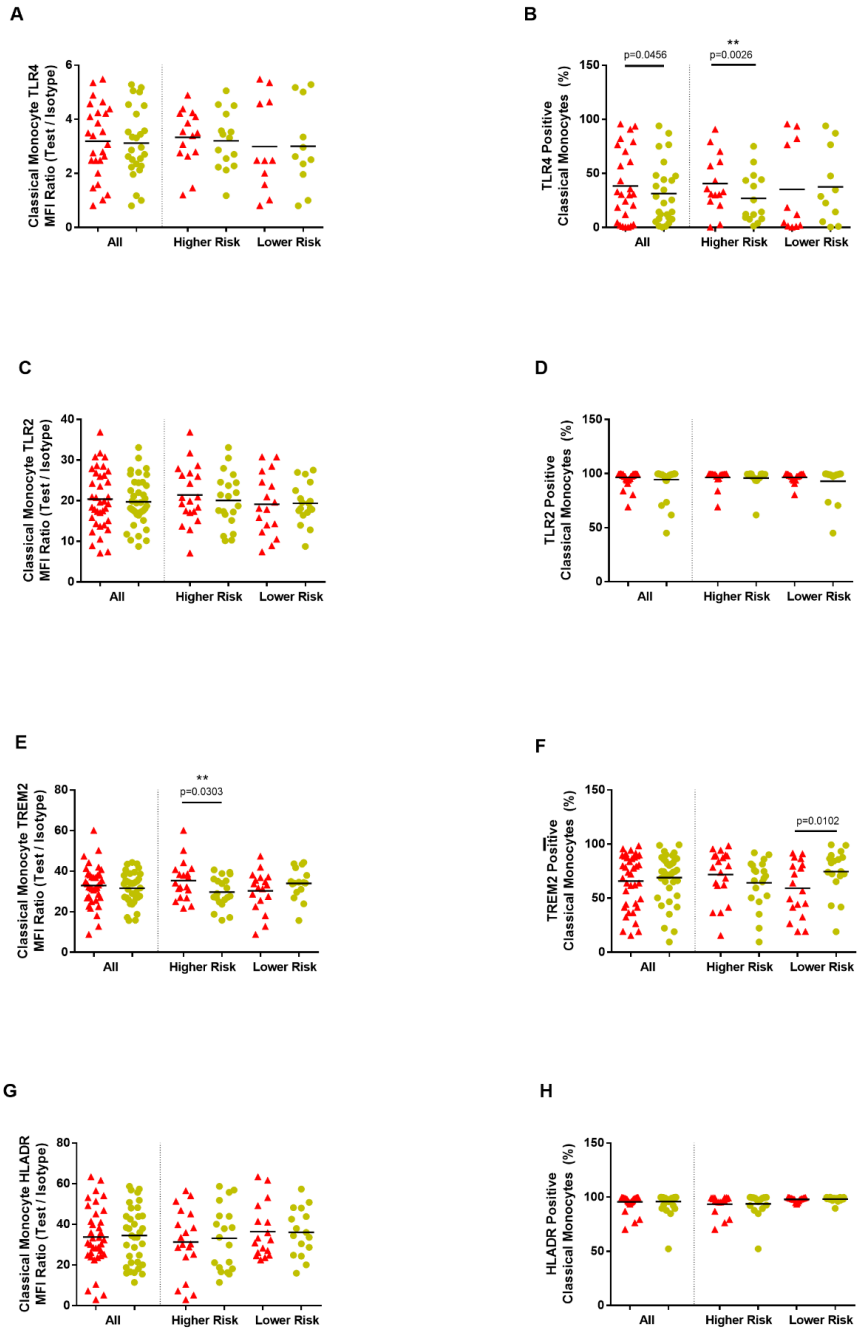

Figure A.5 - Classical monocyte marker expression in Parkinson's disease cases versus paired controls; overall and within risk groups. *Graphs showing Classical monocyte MFI (Median Fluorescence Intensity) ratios (Test/Isotype) ((A), (C), (E), (G)) and percentage Classical monocytes positive ((B), (D), (F), (H)). (Parkinson's disease=red; Controls=yellow). **significance withstood Bonferroni correction for multiple testing within the relevant category.*

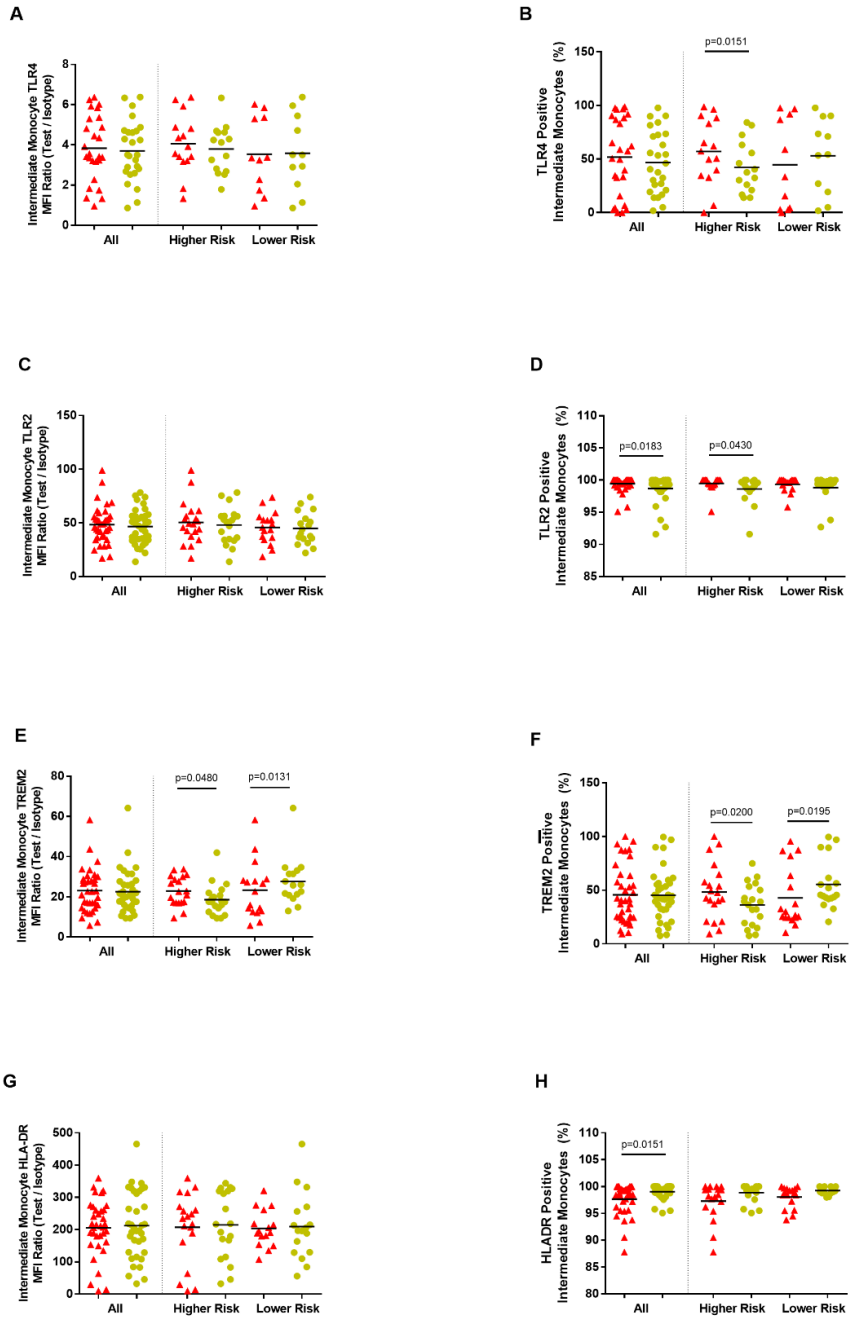

Figure A.6 - Intermediate monocyte marker expression in Parkinson's disease cases versus paired controls; overall and within risk groups. *Graphs showing Intermediate monocyte MFI (Median Fluorescence Intensity) ratios (Test/Isotype) ((A), (C), (E), (G)) and percentage Intermediate monocytes positive ((B), (D), (F), (H)). (Parkinson's disease=red; Controls=yellow). **significance withstood Bonferroni correction for multiple testing within the relevant category.*

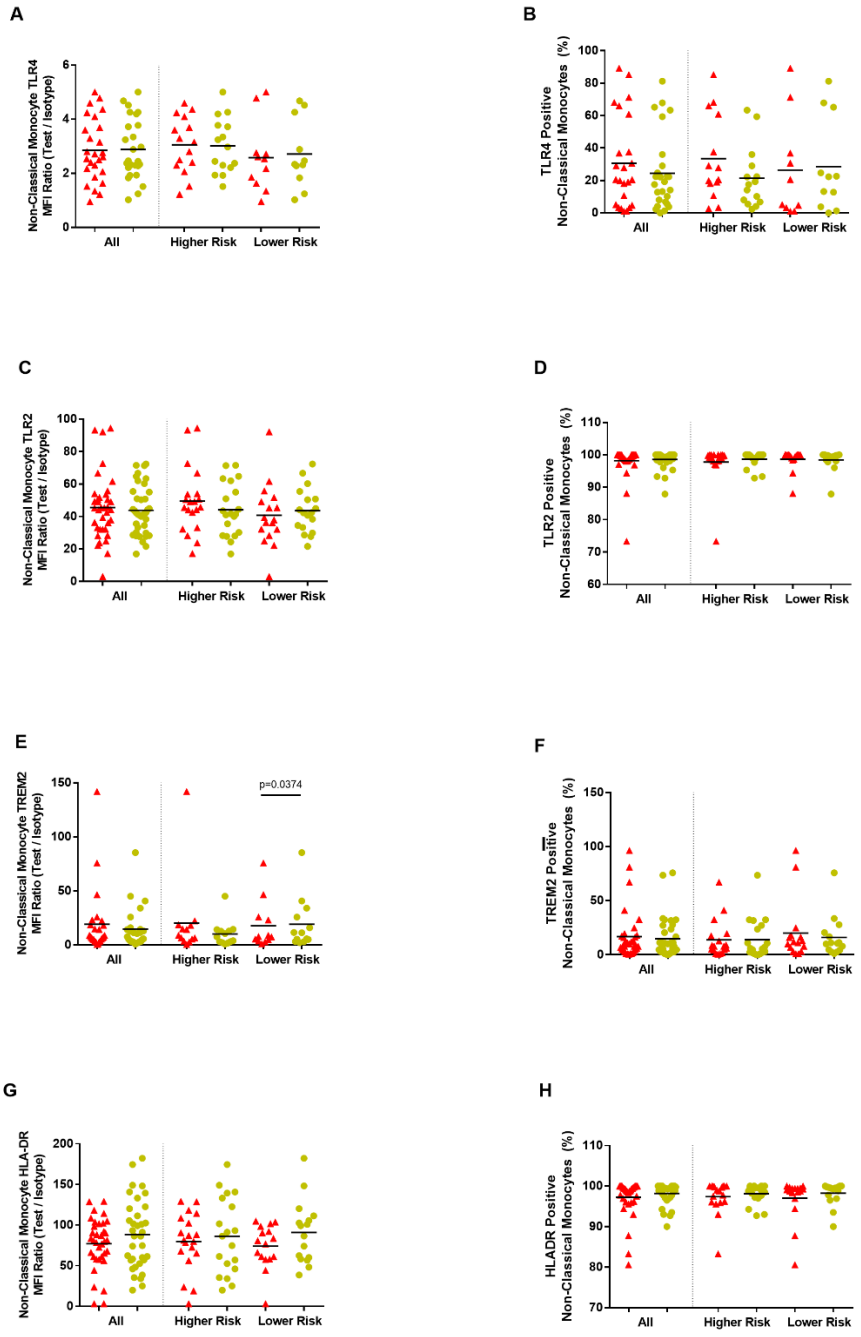

Figure A.7 – Non-Classical monocyte marker expression in Parkinson's disease cases versus paired controls; overall and within risk groups. *Graphs showing Non-Classical monocyte MFI (Median Fluorescence Intensity) ratios (Test/Isotype) ((A), (C), (E), (G)) and percentage Non-Classical monocytes positive ((B), (D), (F), (H)). (Parkinson's disease=red; Controls=yellow). **significance withstood Bonferroni correction for multiple testing within the relevant category.*

3.2 Monocyte markers and associations with clinical and comorbidity variables

Variable (UPDRS motor score)	**Beta Coefficient (B)**	**Significance**	**95% Confidence Interval for B**	
			Lower	**Upper**
Total monocyte HLA-DR	**- 0.308**	**0.022***	**-0.567**	**-0.048**
Age	0.608	0.084	-0.086	1.301
Disease duration	- 1.679	0.359	-5.363	2.005
Levodopa equivalent dose	0.011	0.133	-0.004	0.026
CIRS total score	- 0.902	0.285	-2.594	0.789
(ACE-R score)				
Total monocyte HLA-DR	**0.216**	**0.012***	**0.052**	**0.380**
Age	-0.222	0.314	-0.666	0.221
Disease duration	-0.554	0.640	-2.954	1.846
Levodopa equivalent dose	0.007	0.163	-0.003	0.016
CIRS total score	0.874	0.102	-0.187	1.935
Years of Education	0.431	0.196	-0.236	1.098
(Semantic Fluency)				
Total monocyte HLA-DR	**0.227**	**0.003***	**0.086**	**0.367**
Age	-0.233	0.216	-0.609	0.144
Disease duration	0.894	0.381	-1.162	2.949
Levodopa equivalent dose	0.004	0.328	-0.004	0.012
CIRS total score	0.548	0.219	-0.344	1.440
Years of Education	0.331	0.244	-0.238	0.901

Table A.2 – Monocyte HLA-DR and clinical data - *Results of Linear Regression Analyses with MDS-UPDRS motor score, ACE-R score and Semantic Fluency scores as the dependent variables. CIRS – Cumulative Illness Rating Scale. *p<0.05*

3.3 Serum assay results

Serum MSD Assays

Pro-inflammatory cytokine panel, CRP and alpha-synuclein assays

Serum samples from visit 1, 2 and 3 were used for these assays and equivalent samples from each patient and control pair were analysed within the same experiment. The proteins for which >75% of the samples produced a measurable result across visits (IFN-γ, IL-2, IL-6, IL-8, IL-10, TNF-α, CRP and alpha-synuclein) were included in the analysis.

The average co-efficient of variance for all the assays repeated across the three visits was <1.0. Reliability analysis was performed to calculate the Intraclass correlation coefficient (ICC) estimates and their 95% confidence intervals based on a mean-rating (k=3), absolute-agreement, 2-way mixed-effects model (Koo and Li, 2016). The ICCs for each assay indicated that there was a degree of variability in the results from the three visits (Table A.3). Therefore, the average assay values from the three visits was used for all further analyses of these markers.

Variable	Intraclass Correlation Coefficient (ICC)	95% Confidence Interval	
		Lower	Upper
Serum IFN-γ	0.479	0.201	0.672
Serum IL-2	0.890	0.827	0.933
Serum IL-6	0.442	0.155	0.643
Serum IL-8	0.754	0.635	0.839
Serum IL-10	0.639	0.453	0.769
Serum TNFα	0.777	0.670	0.853
Serum CRP	0.425	0.129	0.632
Serum Alpha-synuclein	0.469	0.196	0.660

Table A.3 -Reliability analysis of serum assays *- Table showing the Intraclass Correlation Coefficients (ICCs) of the serum markers measured at three time points. ICC approaching 1.0 indicates low variability.*

Variable	Group	Parkinson's disease patients	Paired Controls	p
Number (n)	**All**	41	41	
	HR	23	23	
	LR	18	18	
IFN-γ	**All**	6.86 (3.66)	8.63 (4.24)	0.0873
	HR	6.46 (3.32)	9.32 (4.70)	0.0484*
	LR	7.34 (4.07)	7.75 (3.49)	0.7660
IL-2	**All**	0.16 (0.06)	0.17 (0.08)	0.0785
	HR	0.15 (0.06)	0.20 (0.10)	0.0576
	LR	0.15 (0.08)	0.15 (0.07)	0.8653
IL-6	**All**	0.76 (0.42)	0.67 (0.24)	0.1366
	HR	0.86 (0.52)	0.67 (0.25)	0.1069
	LR	0.65 (0.20)	0.66 (0.24)	0.8525
IL-8	**All**	9.84 (2.84)	10.68 (3.24)	0.2604
	HR	9.30 (2.42)	10.24 (1.86)	0.1929
	LR	10.54 (3.23)	11.23 (4.42)	0.6353
IL-10	**All**	0.24 (0.12)	0.26 (0.13)	0.5099
	HR	0.26 (0.13)	0.26 (0.10)	0.8987
	LR	0.22 (0.09)	0.25 (0.16)	0.3692
TNF-α	**All**	2.48 (0.62)	2.66 (0.56)	0.1642
	HR	2.61 (0.67)	2.71 (0.39)	0.5336
	LR	2.31 (0.52)	2.60 (0.73)	0.1928
CRP	**All**	3,797,186.93 (5,798,439.01)	2,672,006.75 (2,091,027.477)	0.6637
	HR	4,591,991.32 (6,751,243.54)	2,953,075.16 (1,974,678.71)	0.7854
	LR	2,825,759.33 (4,360,282.17)	2,328,478,70 (2,233,103.47)	0.6397
Alpha-synuclein	**All**	18,654.48 (6707.89)	33,112.36 (9552.62)	<0.0001**
	HR	18,719.03 (6665.36)	33,629.46 (9084.03)	<0.0001**
	LR	18,567.14 (6969.64)	32,451.61 (10,348.80)	0.0001**

Table A.4 - Summary of serum MSD assay results *(cytokines, CRP and alpha-synuclein). All values indicated are the mean concentration (standard deviation) in pg/ml. Significance (p) indicated is that from paired analysis (parametric paired t-tests - IL-2, IL-6, IL-8, TNF-α; non-parametric Wilcoxon-matched pairs tests – IFN-γ, IL-10, CRP, alpha-synuclein).*

**p<0.05; ** remains significant following correction for multiple testing over all analysed serum markers (12). HR =Higher Risk; LR = Lower Risk.*

3.4 Serum markers and associations with clinical and comorbidity variables

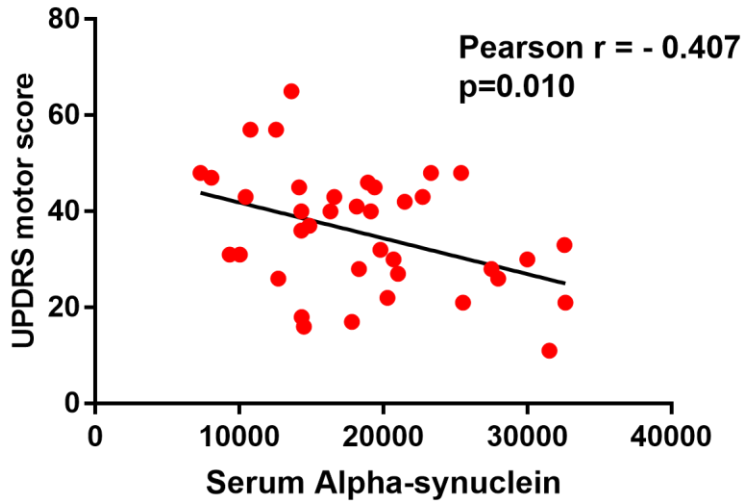

Figure A.8 – Serum alpha-synuclein and motor function *- Scatter plot of serum alpha-synuclein and MDS-UPDRS total motor score.*

Variable	Beta Coefficient (B)	Significance	95% Confidence Interval for B	
			Lower	Upper
Serum alpha-synuclein	-0.001	0.051	-0.001	0.000
Age	0.526	0.124	-0.152	1.204
Disease duration	-1.214	0.502	-4.852	2.424
Levodopa equivalent dose	0.005	0.470	-0.009	0.020
CIRS total score	-0.617	0.467	-2.323	1.089

Table A.5- Serum alpha-synuclein and clinical data. *Linear Regression Analysis with UPDRS motor score as the dependent variable. CIRS – Cumulative Illness Rating Scale.*

3.5 Principal Component Analysis (PCA)

A PCA was run using all participant data on all monocyte markers and the serum markers with uncorrected significant results on overall PD-Control paired analysis. Where more than one measure relating to a factor was significant (e.g. % positive and MFI ratio), the most significant measure for each factor was used for the PCA. Thus, the variables included in the PCA were -: classical monocyte %, monocyte TREM2 MFI ratio, monocyte HLA-DR MFI ratio, TLR2+ monocyte %, TLR4+ monocyte %, serum caspase-1, serum alpha-synuclein, serum endotoxin. The suitability of PCA was assessed prior to analysis. Variables with the lowest Kaiser-Meyer-Olkin (KMO) measures were removed until all the individual KMO measures were greater than 0.55, resulting in the exclusion of TLR4 and HLA-DR from the PCA. The overall KMO

measure for Sampling Adequacy was 0.627. Bartlett's test of sphericity was statistically significant (p=0.001), indicating that the data was likely factorable.

PCA revealed two components that had eigenvalues greater than one and which explained 32.48% and 19.48% of the total variance, respectively. One further component had an eigenvalue > 0.94, explaining a further 15.67% of the variance and visual inspection of the scree plot indicated that three components should be retained. The three-component solution cumulatively explained 67.64% of the total variance. A Varimax rotation with Kaiser normalization was employed to aid interpretability (Table 2).

3.6 Endotoxin and TREM2

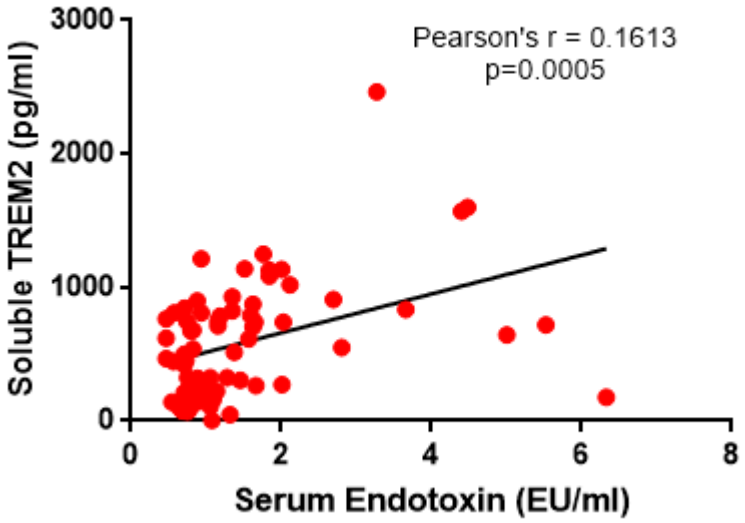

Figure A.9 - Serum endotoxin and soluble TREM2 *- Graph demonstrating a positive relationship between serum endotoxin and soluble TREM2.*

Variable	Beta Coefficient (B)	Significance	95% Confidence Interval for B	
			Lower	Upper
Serum Endotoxin	123.59	0.005	39.80	207.38
Total Monocyte TREM2	-2.52	0.660	-13.96	8.90
Age	15.38	0.083	-2.06	32.84

Table A.6 - Serum endotoxin and soluble TREM2 - *Multiple linear regression analysis with soluble TREM2 as the dependent variable.*

3.7 Alpha-synuclein and Caspase-1

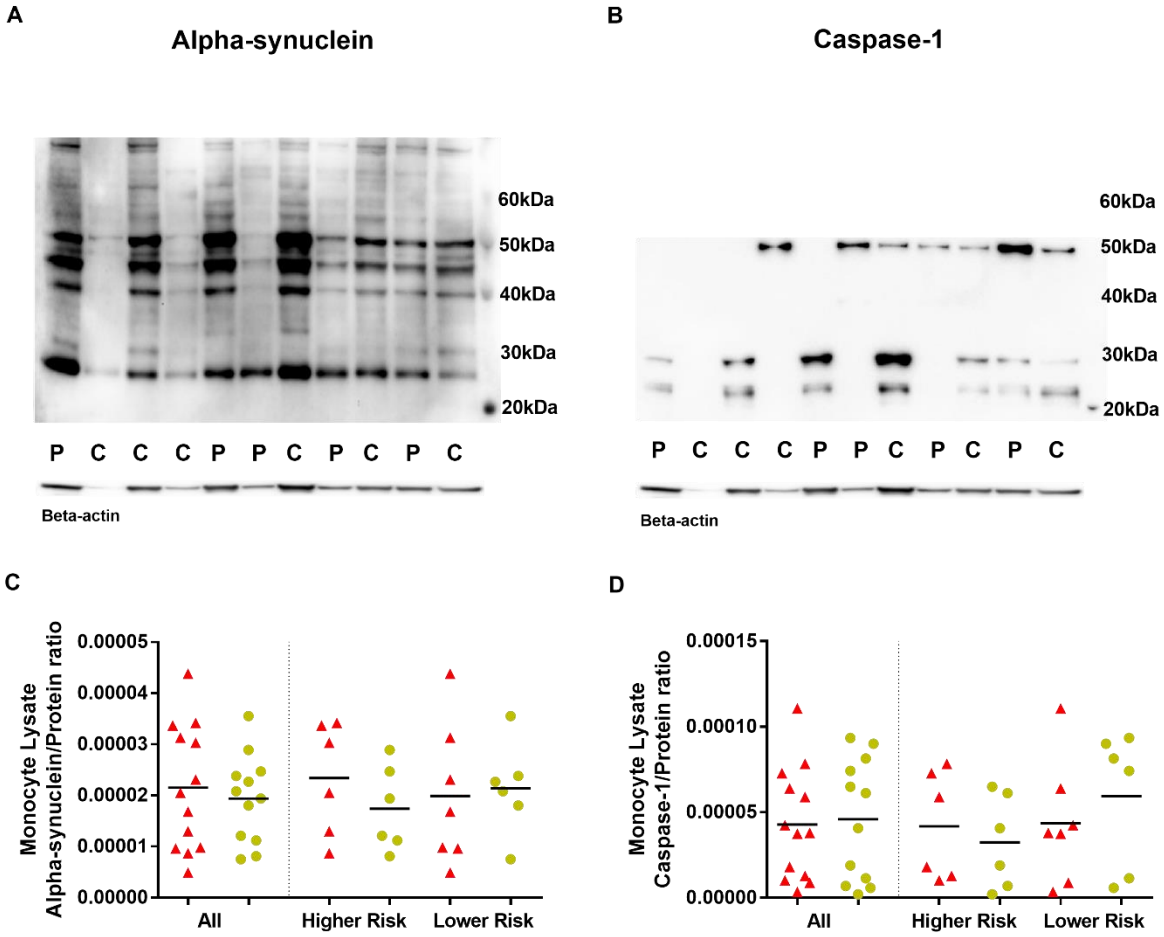

Figure A.10 - Monocyte Lysates *– (A) and (B) Western blots of monocyte lysates for alpha-synuclein (A) and caspase-1 (B), with beta-actin loading control. P=Patient; C=Control. (C) and (D) Monocyte lysate alpha-synuclein (C) and caspase-1 (D) content as a ratio of total protein in patients (red)(n=13) and controls (yellow)(n=12).*

4. Supplementary Data – References

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