CHAPITRE 2: ERYTHROCYTE-DERIVED EXTRACELLULAR VESICLES: A NOVEL, ROBUST AND SPECIFIC BIOMARKER THAT MAPS TO PARKINSON'S DISEASE STAGES

2.1 Résumé

Contexte La production de VE est une caractéristique omniprésente des cellules eucaryotes. Cependant, des conditions pathologiques peuvent affecter leur formation et leur constitution. Nous avons cherché à caractériser la nature, le profil et la signature protéique des VE dans le plasma de deux cohortes indépendantes de patients atteints de la MP et corréler les résultats avec des mesures cliniques de la maladie. **Méthodologie** Les VE ont été recueillis auprès de grandes cohortes de patients souffrant de la MP (n = 60 ; Controls, n = 37) et de patients souffrant de la maladie d'Huntington (Pré-manifeste, n = 11 ; manifeste, n = 52 ; Contrôles, n = 55) - pour un total de 215 participants. – Ils ont été analysés de manière exhaustive grâce à la cytométrie de flux, la microscopie électronique et la protéomique. **Résultats** Nous avons découvert qu'il existe une liaison de type affine entre le nombre de VE dérivés d'érythrocytes (VEE) et l'échelle clinique, UPDRS. Nous avons observé que les patients atteints de la MP au stade précoce — caractérisés par un score UPDRS inférieur à 37 — ont montré une augmentation linéaire des VEE par rapport à l'état pathologique (corrélations = 0,82), avec un profil presque identique chez les patients modérés avec des scores UPDRS compris entre 37 et 75 (corrélations = 0,873). Ces résultats sont obtenus grâce à un ajustement pour certaines comorbidités. Nous n'avons pas observé ces résultats chez les patients atteints de la maladie d'Huntington, ce qui met en évidence la spécificité de nos résultats vis-à-vis la pathologie de la MP. D'autres analyses protéomiques des VEE ont permis d'identifiés 8 protéines dont l'expression a été significativement modifiée chez les patients atteints de la MP aux stades précoce et modéré. **Conclusion** Nous avons découvert un nouveau biomarqueur qui différencie les étapes de la MP en fonction de la quantification des VEE et de leur cargaison unique de protéines.

Erythrocyte-derived extracellular vesicles: A novel, robust and specific biomarker that maps to Parkinson's disease stages

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2.2 Abstract

Background The production of extracellular vesicles (EV) is a ubiquitous feature of eukaryotic cells, but pathological events can affect their formation and constituents. We sought to characterize the nature, profile and protein signature of EV in the plasma of two independent cohorts of Parkinson's disease (PD) patients and how they correlate to clinical measures of the disease. **Methods** EVs were collected from large cohorts of PD (n=60; Controls, n=37) and Huntington's disease (HD) patients (Pre-manifest, n=11; manifest, n=52; Controls, n=55) - for a total of 215 participants - and exhaustively analyzed using flow cytometry, electron microscopy and proteomics. **Results** We discovered that the number of EV derived from erythrocytes (EEV) was strongly correlated to two different disease stages as defined using the Unified Parkinson Disease Rating Scale (UPDRS). In particular, we observed that mild PD patients – characterized by a UPDRS score lower than 37 – showed a linear increase of EV with respect to disease state (correlations = 0.82), with a near identical pattern in moderate patients with UPDRS scores ranging between 37 and 75 (correlations = 0.873). We did not observe this in patients with HD, highlighting the specificity of our findings to PD process. Further proteomic analyses of the EV identified 8 proteins whose expression was significantly altered in both mild and moderate PD patients. **Conclusion** We have discovered a novel biomarker that maps onto stages of PD based on the quantification of EV shed from erythrocytes and their unique protein cargo.

2.3 Introduction

There is no doubt that over the last few years, there has been a dramatic increase in new findings that have fundamentally transformed our understanding of neurodegenerative diseases, in particular of Parkinson's disease (PD). One of these has been the heterogeneity of the disease that has hindered the development of useful biomarkers, as well as the successful translation of novel treatments. This new understanding of the different clinical profiles seen in populations of PD patients has begun to translate into a redefinition of PD, as is evidenced by new diagnostic criteria released by the International PD and Movement Disorder Society (MDS) (Berg et al., 2014). This task force has also highlighted the need to identify reliable biomarkers that can help in the diagnosis, stratification and tracking of this condition.

In a novel approach to develop such biomarkers, we exhaustively studied circulating extracellular vesicles (EV), known to mediate cell-to-cell communication in both physiological and disease conditions (Quek and Hill, 2017). Their rich protein cargo, cell type signature, and availability in bodily fluids such as blood plasma make them very attractive biomarkers (Porro et al., 2015). We therefore characterized the nature, protein signature and profile of EV in the plasma of two independent cohorts of PD patients and assessed their behaviour in relation to disease states. Among the most important clinical challenges is the assessment of drug efficacy, and more specifically in complex neurodegenerative diseases with heterogeneous populations of patients. In PD, for example, some patients evolve to an early dementia while others remain cognitively normal throughout their illness and although we have some clinical and underlying genetic clues as to why this may happen, we have no way of accurately predicting or following the course of these different subtypes of PD. This is critical if we are to bring specific targeted therapeutics to the clinic to treat different aspects of the disease, especially therapies that are designed to be disease modifying which ideally should be targeted to the more rapidly progressive forms of PD.

2.4 Materials and methods

2.4.1 Ethics statement and participant recruitment

Institutional review boards approved this study (CHU de Québec, #A13-2-1096; CHUM, #14.228; Cambridge Central Regional Ethics Committee, REC #03/303 & #08/H0306/26; and Cambridge University Hospitals Foundation Trust Research and Development department, R&D #A085170 & #A091246) in accordance with the Declaration of Helsinki, and written informed consents were obtained from all participants.

Blood samples were collected from 2 cohorts of patients with PD [one in Cambridge UK; one in Quebec Canada] as well as an independent cohort of Huntington's disease (HD) individuals [collected in Montreal, Canada] along with age- and sex-matched healthy Controls (**Table 2.1**). In the case of PD patients, the UK PDS Brain diagnostic criteria were used which gives a diagnostic accuracy of 98.6% when applied by movement disorder specialists (Massano and Bhatia, 2012; Hughes et al., 2002). Their clinical evaluation included the Unified Parkinson Disease Rating Scale (UPDRS), Hoehn and Yahr (H&Y) staging, the Mini Mental State Examination (MMSE), the Addenbrooke's Cognitive Examination (ACE) and the Beck Depression Inventory (BDI). In the case of the HD patients, their Unified Huntington Disease Rating Scale (UHDRS), Total Functional capacity (TFC) and calculated values for burden of disease (BDS) were all collected and diagnosis was confirmed by genetic testing. All clinical evaluations were conducted within 6 months of the blood collection. Participants were further asked to fill out a questionnaire related to health issues and medication and a full blood count was performed in all patients on the day of blood sampling. Medications were converted into levodopa equivalent daily dose (LEDD) using common calculator tools. It should be noted that blood sampling was conducted by the exact same team of investigators, following identical procedures in both UK and Canada.

2.4.2 Preparation of platelet-free plasma and EV labeling

Citrated blood was centrifuged twice for 15 minutes at 2500g at room temperature. Platelet-free plasma (PFP) was harvested and stored at -80°C within 2 hours of sampling following

previously published guidelines (Lacroix et al., 2012). For specific details on EV labeling, please refer to supplementary material.

In all cases, the control samples came from healthy participants and the blood was collected at exactly the same time as the PD and HD patients in the UK and Canadian clinics. Every blood sample was processed immediately to avoid release of non-physiological EV. For analyses relating to EV in the plasma, all blood samples were centrifuged twice at 2500g to enable PFP recovery. The plasma was fractionated into 3 aliquots per individual and frozen immediately following processing (maximum of 2 hours following blood collection). Blood samples from Controls and PD were collected in parallel and laboratory analyses were blinded to participant status.

2.4.3 Flow cytometry quantification

For EV quantification, we used a FACS Canto II Special Order Research Product equipped with a forward scatter (FSC) coupled to a photomultiplier tube (FSC-PMT) and a small particle option. Flow cytometer performance tracking was carried out daily using the BD cytometer setup and tracking beads (BD Biosciences, San Jose, CA, USA). The size of the EV was determined using fluorescent silica beads of 100, 500 and 1000nm (**figure S2.1**). The settings for the EV detection were determined as previously described using a threshold of 200 for SSC (also see **figure S2.1**) (Rousseau et al., 2015). For specific details plasma-related EV quantification, please refer to supplementary material.

2.4.4 Production and purification of EEV

Blood was collected in heparin tubes and centrifuged for 10 minutes at 282g at room temperature. Blood cells were washed first in PBS-2%FBS, then with 0.9% sodium chloride solution and centrifuged for 10 minutes at 750g. To avoid leukocyte and/or platelet contamination, the buffy coat and upper fraction of erythrocytes were removed. To preserve erythrocytes, two volumes of glycerolyte 57 solution (57% glycerol, 142mM sodium lactate, 1mM KCl, 25mM sodium phosphate pH 6.8) were added to the pellet and stored at -80°C. For the production of EEV, red bloods cells were thawed and EV production was induced as

previously described (Minetti et al., 2004). For specific details on EEV purification, please refer to supplementary material.

2.4.5 C-reactive protein, free hemoglobin and α-synuclein quantification

The concentrations of C-reactive protein (CRP) and free hemoglobin were determined in the PFP of all donors using the RayBio Human CRP ELISA Kit (RayBiotech, Norcross, GA, USA) and the Hemoglobin Human ELISA kit (Abcam, Toronto, ON, Canada). To quantify α-synuclein (α-Syn) in erythrocytes and EEV, we used the human α-Syn ELISA kit (ThermoFisher Scientific, Waltham, MA, USA). Absorbance values were measured at 450nm using a multi-detection microplate reader (Synergy HT; BioTek; Winooski, VT, USA). All ELISA tests were performed according to the manufacturer's instructions.

2.4.6 Scanning electron microscopy

For the visualization by scanning electron microscopy, EEV were prepared as previously described (Duchez et al., 2015). For specific details on EEV preparation, please refer to supplementary material.

2.4.7 Transmission electron microscopy

For the transmission electron microscopy, EEV were prepared and observed as previously described (Duchez et al., 2015). For specific details on EEV preparation and labelling, please refer to supplementary material.

2.4.8 Mass spectrometry analysis and label free protein quantification

The initial proteomic analyses were performed on 3 pools of 3 blood samples of each group. Following this, we undertook a completely new set of analyses to develop a method allowing us to isolate hemoglobin and better investigate the EEV proteome. For this, EVs from an additional 4 individuals per group (Control, mild PD and moderate PD) were prepared as described above. For each individual, 25µg of protein sample, according to Bradford protein

assay, were migrated onto an electrophoresis gel 4-12% Bis-Tris to separate hemoglobin from higher proteins. Following gel staining using Sypro Ruby (Thermo Fischer Scientific), the 12kDa band corresponding to the hemoglobin size was cut out and the remaining part of the gel further fractioned into 7 slices, exposed to trypsin digestion and peptide extraction on a MassPrep liquid handling robot (Waters, Milford, USA) according to the manufacturer's specifications and the protocol of Shevchenko et al. with the modifications suggested by Havlis et al. (Shevchenko et al., 1996; Havlis et al., 2003). For specific details on protein analysis purification, please refer to supplementary material. Spectra of peptides were searched against a human protein database (Uniprot Complete Proteome, taxonomy Homo sapiens – 83512 sequences) using the Andromeda search engine included in MaxQuant software version 1.5.5.1 (Cox and Mann, 2008). MaxQuant was also used to validate proteins and peptides at a 1% False Discovery Rate using a target/decoy database search as well as to undertake a Label Free Quantification of the identified proteins using the 'match between runs' option.

2.4.9 Statistical analyses

All statistical analyses were performed by a quakified statistician and details pertaining to these analyses for each experiments are provided in the supplementary material.

2.5 Results

This study involved two cohorts of PD patients (*n*=60; *n*=36 Cambridge; *n*=24 Quebec) and HD patients (*n*=63; Quebec) at all stages of diseases along with age- and sex-matched healthy Controls (*n*=37 for PD; *n*=55 for HD) (**Table 2.1**) giving a total of 215 participants. First, we evaluated the diagnostic value of EV for PD and found a notable increase in the mean number of EEV in PD patients with respect to their age- and sex-matched healthy Controls (p=0.0319; **figure 2.1A**). A closer look at the data distribution, however, revealed that only 5 patients were responsible for this significant difference (**figure 2.1A**, red inset). Further investigation using a diagnostic accuracy test, revealed that the relevant proportion of the area under the Receiver Operating Characteristic (ROC) curve was 0.508 (data not shown), implying that

the number of EEV could not solely be used as a discriminant between PD patients and healthy Controls. This was also confirmed by performing a Bayesian analysis of the data demonstrating that the proposed predictor of the onset of PD would be correct in less than 1% of the cases (data not shown). We obtained similar result when we separated the patients into mild and moderate states according to their UPDRS scores (data not shown).

We next evaluated the potential of EEV to map onto different PD disease stages. Analyses revealed striking correlations between the number of vesicles derived from the red blood cells of PD patients and their total UPDRS (**figure 2.1B**). Statistical linear regression analysis was performed for each group and the R^2 values obtained demonstrated that in both cases, at least 87% of the variation in the total number of EEV/erythrocytes was due to the variation of the UPDRS (**figure 2.1B**). Moreover, the results are significant with respect to the *p* values obtained for each fit, since they fall below the 5% confidence level. Hence, the statistical tests on EEV counts uncovered a clear cut-off point between mild and moderate patients, which could not be accounted for by medication, as there was no correlation with the patients LEDD (**figure S2.2A-B**). Additional analyses on the number of EEV in relation to other clinical measures including H&Y stage, BDI, MMSE and ACE found no significant correlations (data not shown).

In the HD cohort, the total number of EEV between pre-manifest and manifest HD was similar to that seen in their age- and sex-matched healthy Controls (**figure 2.1C**). In contrast to PD, correlation analyses failed to reveal an association between the number of EEV and HD stage using the UHDRS score (**figure 2.1D**). Based on this, all our subsequent analyses focused only on the PD cohort. It should also be noted that total blood counts did not indicate any differences in the number of endothelial cell-, platelet-, monocyte- and granulocyte-derived EV in the platelet-free plasma (PFP) in PD (**Table S2.1**) and HD patients (**Table S2.2**) when compared to their respective control cohorts. Additionally, at the start of the study and as a precautionary measure, full blood count (**figure S2.3A-C**) and C-reactive protein (**figure S2.3D**) quantifications were obtained for all participants which revealed no differences between groups. We did, however, observe a significant increase in EEV concentration in the PFP of individuals with diabetes and those suffering, or having suffered,

from cancer (data not shown) and thus these participants were excluded from our analyses. PFP samples with elevated free hemoglobin (>45 000ng/ml), potentially due to hemolysis during blood sampling, were also excluded from further EEV-related analyses (**figure S2.3E**) which account for the small discrepancies between the total number of participants initially recruited and those contained in each analysis.

Having established that EEV counts map to disease state in PD, we aimed to assess whether α-Syn – which is not only the main component of Lewy bodies but is highly expressed in most blood cells – was differentially expressed in normal vs. diseased conditions. For this, we opted to use scanning electron microscopy, but this did not reveal any morphological changes between resting and activated erythrocytes in either condition (**figure 2.2A**). We further used transmission electron microscopy to quantify the number of EEV containing α-Syn and phosphorylated (serine 129) forms of the protein but again no significant differences between PD patients and age- and sex-matched healthy Controls (**figure 2.2B-C**) were observed. Quantified α-Syn levels in EEV from PD patients and Controls using commercial ELISA kits corroborated these results (**figure 2.2D**).

Since our combined quantification studies (ELISA and transmission electron microscopy) suggested that α-Syn levels could not be used as a blood marker of disease, we sought to obtain the specific protein signature of EEV from mild and moderate PD patients (with respect to the UPDRS scores) and their age-matched Controls. Given the significant amounts of hemoglobin within erythrocytes that could mask the true nature of the protein signature in EEV, we performed a label free quantitative proteomic analysis by nanoLC/MSMS (Wither et al., 2016) using two distinct approaches: with and without hemoglobin (**figure S2.4A**). By removing hemoglobin, we identified a total of 818 proteins in comparison with 356 when we did not undertake this methodological step (refer to **Table S2.3** for complete list of proteins) – a modification which is clearly needed to give a much more accurate evaluation of the protein content of EEV. Additionally, a Gene Ontology enrichment analysis on the 'Cellular Component' ontology on the two sets of identified proteins in comparison with the whole human proteome, revealed that our samples are enriched with elements associated with 'vesicles' and 'hemoglobin complex' (**figure S2.4B**).

Out of the 818 proteins identified in the proteome of EEV, 8 had their expression significantly modified according to the different stages of PD (**figure 2.3A-B**). Hierarchical clustering, coupled to a heatmap, allowed us to group individuals according to stages of disease (Control, mild PD and moderate PD) and provided compelling evidence that the 8 proteins identified could also be grouped into three categories. Proteins of group I were highly and predominantly expressed in Controls, proteins of group II were highly and predominantly expressed in mild PD patients and proteins belonging to group III were highly and predominantly expressed in moderate PD patients (**figure 2.3C**). This data set was further confirmed by volcano plots (**figure S2.5**). Of interest, two proteins of group I are associated with the regulation system of the cell (ABHD14B, NADSYN1) and one protein significantly expressed in moderate PD patients (ATP5A1) is involved in the regulation of mitochondrial ATP production.

2.6 Discussion

We have identified a novel biomarker that maps to different states of PD, using two cohorts of patients and a total of 215 participants, based on the quantification of EV shed from erythrocytes. In particular, we have shown a remarkable correlation between the number of EEV and the clinical expression/stages of PD using the standard UPDRS measure. Finding this level of correlation with such a heterogeneous disorder highlights the robustness of the biomarker we have identified. This is also unique to PD as no such changes were seen in a second neurodegenerative disorder of the brain, HD. As these EVs contain a unique protein cargo, we surmise that the EVs and their content will help us understand the basis for the different disease stages in PD, with the possibility that the disease pathogenesis changes at a critical point in its clinical course.

It has previously been reported that EV derived from the cerebrospinal fluid contain α-Syn, its associated pathogenic species as well as the LRRK2 protein (Kunadt et al., 2015; Fraser et al., 2013). However, routinely collecting cerebrospinal fluid presents a challenge given its invasive nature while plasma is a very accessible fluid that further allows real time

monitoring, and thus has been more extensively studied from a biomarker perspective. Indeed, α-Syn expression has been reported in platelets, leukocytes and red blood cells, with some studies suggesting that their levels correlate with disease duration (Behari and Shrivastava, 2013; Fuchs et al., 2008; Kim et al., 2004; Li et al., 2002b; Nakai et al., 2007). However, none of these studies have shown that blood cells, or EV derived from blood cells, represent a robust biomarker of disease (see summary **figure 2.4**) (Nakai et al., 2007; Wang et al., 2015; Barbour et al., 2008; Pretorius et al., 2014; Nikam et al., 2009; Sudha et al., 2003; Bartels et al., 2011; Fauvet et al., 2012; Araki et al., 2016; Helferich et al., 2015; Renella et al., 2014; Abd-Elhadi et al., 2015). Despite the fact that a number of groups have reported the presence of EV in various bodily fluids of PD patients (**figure 2.4**) (Kunadt et al., 2015; Fraser et al., 2013; Emmanouilidou et al., 2010; Danzer et al., 2012; Alvarez-Erviti et al., 2011; Melachroinou et al., 2013; Grey et al., 2015; Kong et al., 2014; Tsunemi et al., 2014; Shi et al., 2014; Tomlinson et al., 2015; El-Agnaf et al., 2006; Stuendl et al., 2016; Ho et al., 2014; Zappulli et al., 2016), we provide clear evidence that EEV can serve as a biomarker in PD.

Although staging of PD is often done using the H&Y clinical scale, we sought to use the UPDRS given its greater sensitivity – an approach that has recently been validated (Martínez-Martín et al., 2015). Using these scores, we found that mild PD patients – with a UPDRS score lower than 37 – have an increased number of EV (correlations = 0.886); and that the exact same pattern then repeats itself with patients who had UPDRS scores between 37 and 75 (correlations = 0.873) with a marked resetting of the biomarker between these two stages. When comparing individual patients from both correlation curves, LEDD did not seem to account for the differences, as there was no correlation between the LEDD and the number of EEV. At this stage, it is unclear why there is this split in correlation around the UPDRS score and EEV counts. Building on these cross-sectional findings, ongoing longitudinal studies in larger patient cohorts, funded by the Weston Brain Institute, will allow to further investigate this.

Another important aspect of the test we have developed is its potential to be easily adopted into the clinic. Most of the tests currently developed for biomarker use in patients are time

consuming and require appreciable quantities of biological samples. Our biomarker employs a user-friendly clinical test which has the potential to be easily automated using limited volumes of blood (few microliters). Finally, while proteomic studies of EEV have been previously reported (Bosman et al., 2008, 2012), their description was limited to 270 proteins (Shi et al., 2014). We herein report on an improved method to perform this type of analyses in blood samples by removing hemoglobin; a large protein that can easily mask other ones within a protein signature. Indeed, the high dynamic range of protein concentrations in erythrocytes and their EEV, due to the abundance of hemoglobin, decreases the capacity of the mass spectrometer to detect signals corresponding to low abundance proteins. Analyzing the hemoglobin, separately from the other proteins of other molecular weights allowed us to go deeper into the EEV proteome, identifying 129% more proteins than in the initial analysis. Additionally, it is important to reiterate that our proteomic analyses were performed using very stringent parameters, which included a minimum of 2 peptides per protein and p values lesser than 0.05 with an absolute z score of 1.96, corresponding to values outside of the 95% confidence range. Finally, our volcano plots and heatmap analyses revealed high specificity of the identified proteins for each group with nodes ranging between 80 and 90%. With this, reproducibility of our proteomics data is high despite the smaller sample size (analyses performed on 3 pools of 3 samples per group and subsequently on 4 individual samples per group).

Taken together, the identification of this new EEV biomarker has the potential to 1) help in the diagnosis of PD especially at a stage of disease when disease modifying therapies could have their biggest impact, 2) lead to a useful marker that tracks disease course, 3) provide a therapeutic target or marker of disease modification in the development and testing of novel disease modifying therapies and 4) offer new insights into disease pathogenesis and how it changes through the disease stages, which in turn has important therapeutic implications.

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Conflicts of Interest

F.C., E.B., S.L. and I.S.-A. have filed a patent on the use of EEV as a biomarker in PD. J.L.P., R.L., J.P., N.C., F.R.-D., A.T.V., S.L.M., A-C.D., A.D., S.L., N.D., M.L., S.C. and R.A.B. declare no conflicts of interest.

Table 2.1. Participant clinical information

		PD cohort				
	CTRL	PD Patients – Stages of disease				
		Unknown	Mild	Moderate	Severe	*p* value
n	37	7	12	33	8	
Age	66.8	69.8	66.7	71.1	75.0*	**0.04**
Gender F (M)	18 (19)	1 (6)	6 (6)	16 (17)	0 (8)	**0.05**
Disease severity						
Hoehn & Yahr (*n*)			1 ± 0.3 (12)	2 ± 0.2 (33)	3 ± 0.5 (8)	**<0.0001**

UPDRS (*n*)			38 ± 11 (6)	52 ± 19 (17)	73 ± 20 (6)	**0.02**
BDI (*n*)			3 ± 2 (6)	4 ± 2 (17)	13 ± 7 (4)	**0.03**
MMSE (*n*)			29 ± 2 (7)	29 ± 1 (19)	26 ± 3 (6)	**0.01**
ACE (*n*)			96 ± 4 (6)	92 ± 7 (17)	84 ± 14 (6)	0.13
Comorbidities						
Depression	3	1	2	1	2	0.29
Cancer	5	0	3	4	1	0.64
Diabetes	2	0	0	1	2	0.10
Hypertension	10	1	2	10	3	0.76
Hypercholesterolemia	5	0	1	6	1	0.73
Asthma	3	1	1	5	0	0.71
Allergies	2	0	2	6	2	0.28

HD cohort

	CTRL	HD Patients – Stages of disease						
		Pre-HD	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	*p* value
n	55	11	15	13	12	10	2	
Age	55.0	37.5	53.1	54.2	58.3	58.1	55.5	**0.02**
Gender F (M)	31 (22)	6 (5)	5 (10)	4 (9)	8 (4)	7 (3)	1 (1)	0.26
Disease severity								
CAG (*n*)	28.3 (3)	41.1 (10)	42.3 (13)	42.6 (12)	43.7 (7)	44.3 (7)		**<0.001**
UHDRS (*n*)		2.7 (11)	15.7 (14)	34.5 (11)	42.9 (12)	55.9 (10)	67.5 (2)	**<0.001**
TFC (*n*)	13 (16)	13 (11)	12.5 (15)	7.8 (13)	4.3 (12)	1.6 (10)	0 (2)	**<0.001**
BDS (*n*)		206 (10)	337 (13)	356 (12)	442 (7)	465 (7)		**<0.001**
Comorbidities								
Depression	8	1	1	3	6	4	1	**0.0497**
Cancer	0	0	0	0	0	0	0	
Diabetes	3	1	1	1	1	1	0	0.99
Hypertension	4	1	2	1	1	2	0	0.92
Hypercholesterolemia	8	1	1	0	0	1	0	0.32
Asthma	0	0	1	0	0	0	0	0.65
Allergies	3	0	2	2	0	0	0	0.33

2.8 Tableau

Table 2.1. Participant clinical information. (**PD cohort**) Disease severity levels in relation to the H&Y scale (score): Mild (1-1.5); Moderate (2-2.5); Severe (3-3.5). *$p < 0.05$ vs. CTRL. Statistical analyses were performed using a Welch ANOVA followed by Dunnett's multiple comparison test. Disease severity was evaluated within 6 months of blood sampling. Comorbidities were determined from medical information reported by the participant or caregiver. Cancer refers to participant having suffered from cancer in the past. (**HD cohort**) Disease severity levels in relation to the TFC scale: Stage 1 (11-13); Stage 2 (7-10); Stage 3 (3-6); Stage 4 (1-2); Stage 5 (0). Disease severity was evaluated within 6 months of blood sampling. Comorbidities were determined from medical information reported by the participant or caregiver. **Abbreviations**: ACE, Addenbrooke's Cognitive Examination; BDI,

Beck Depression Inventory; BDS, Burden of Disease Score; CTRL, Control; HD, Huntington's disease; H&Y, Hoehn and Yahr; MMSE, Mini-Mental State Examination; PD, Parkinson's disease; UHDRS, Unified Huntington's Disease Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale; TFC, Total Functional Capacity.

2.9 Figures

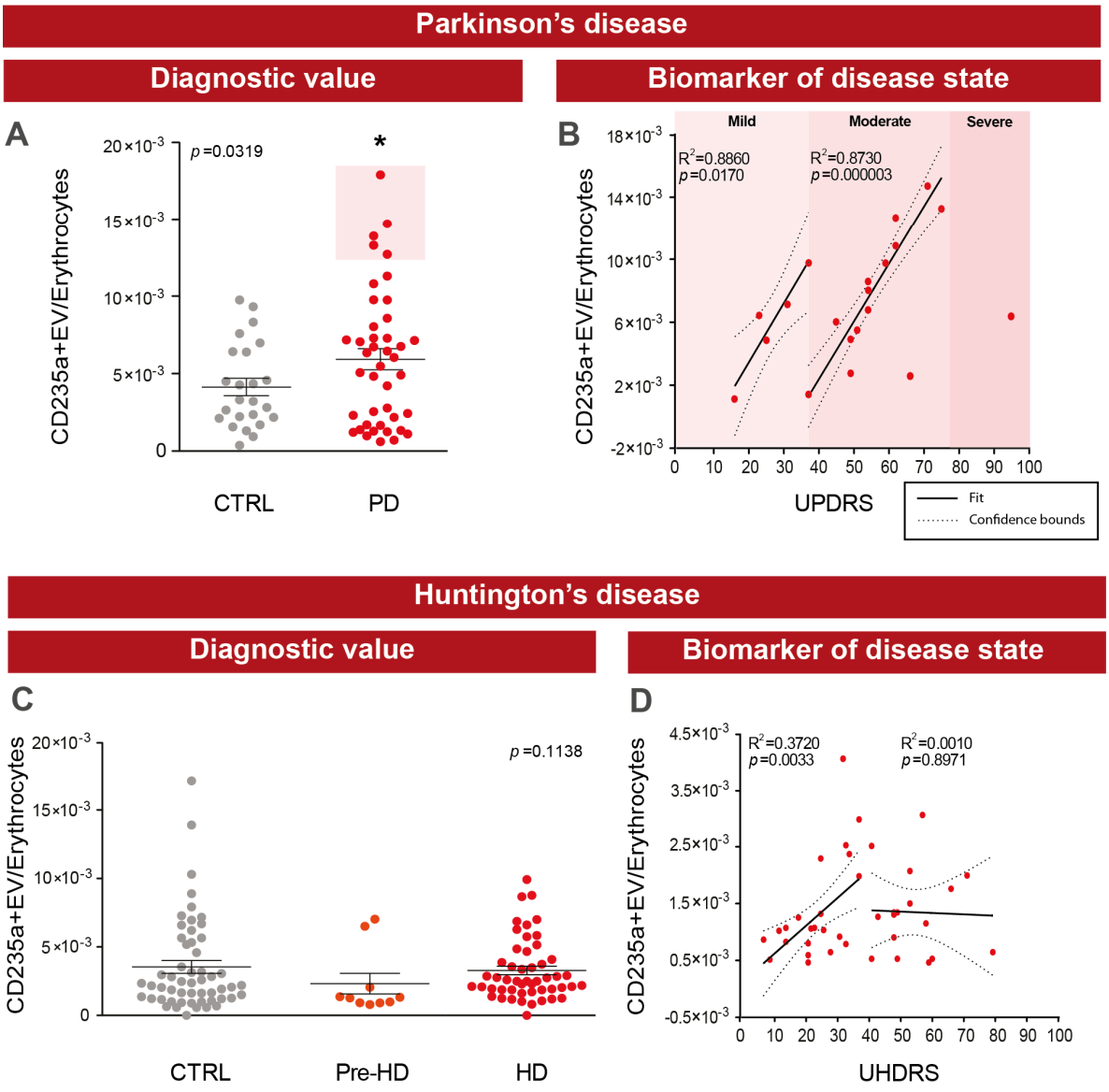


Figure 2.1. EEV: a robust biomarker of PD stage. **A**. Distribution plots of CD235a+ EV/total number of erythrocytes uncovered a significant group difference between PD and healthy sex- and age-matched CTRL (PD, *n*=42; CTRL, *n*=24) although only a small number of patients (*n*=5) accounted for this difference. **B**. Robust correlations between the number of EEV/total number of erythrocytes and UPDRS scores (PD, *n*=20, from the UK cohort exclusively since the Quebec cohort did not have recent UPDRS scores available), displayed a clear split between mild and moderate patients, supporting the potential of EEV to act as a biomarker for disease stage. **C**. Distribution plots of CD235a+/total number of erythrocytes

revealed no differences between HD and healthy sex- and age-matched CTRL (HD, *n*=50; Pre-HD, *n*=10; CTRL, *n*=52) and **D**. no statistically significant correlations were found between the number of EEV/total number of erythrocytes and UHDRS scores (HD, *n*=42). This argues in favor of EEV being a specific biomarker for PD. Distributions were determined using unpaired t-test with Welch's correction (PD) or one-way ANOVA (HD). Correlations were determined using Pearson's correlation, $*p<0.05$. **Abbreviations**: CD235a, glycophorin A; CTRL, Controls; EEV, erythrocyte-derived extracellular vesicle; EV, extracellular vesicle; HD, Huntington's disease; PD, Parkinson's disease; Pre-HD, Pre-manifest; UHDRS, Unified Huntington's Disease Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale.

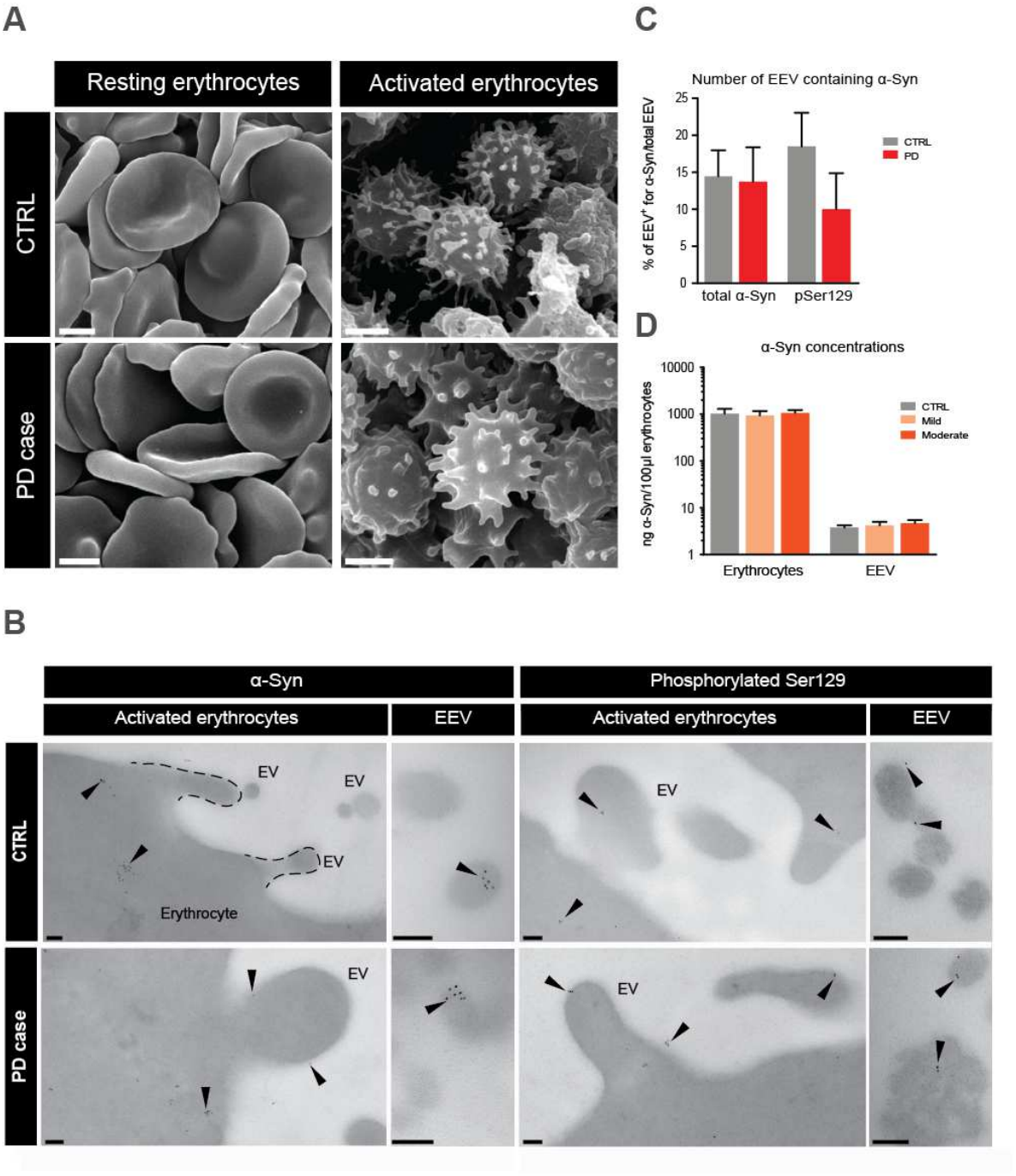
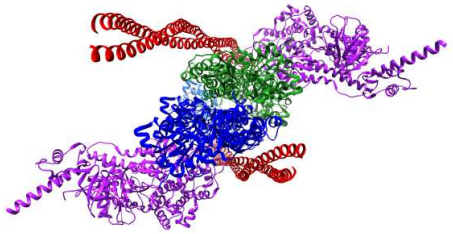


Figure 2.2. Detection of normal and phosphorylated α-Syn in EEV. **A**. Representative scanning electron micrographs of resting and activated erythrocytes (treated with calcium ionophore A23187 to generate EEV) in both PD patients and healthy sex- and age-matched CTRL. Scale bar: 2µm. **B**. Representative transmission electron microscopy images of immunogold labeling for α-Syn and α-Syn pS129 in activated erythrocytes and EEV (some examples delineated by dotted lines). Arrowheads point to positive immunolabeling for either α-Syn or α-Syn pS129. Scale bar: 100nm. **C**. Quantification of α-Syn in EEV as detected by

transmission electron microscopy and expressed as the percentage of EEVs positive for α-Syn/total number of EEV in healthy sex- and age-matched CTRL and PD patients (*n*=100 erythrocytes sampled in *n*=3 CTRL and *n*=3 PD). **D**. Quantification of α-Syn in EEV by ELISA assay in healthy sex- and age-matched CTRL, mild and moderate stage patients (*n*=4 erythrocytes per group; *n*=13 EEV per group) revealing the absence of measurable changes in α-Syn levels between PD and healthy sex- and age-matched CTRL. Statistical analyses were performed using a Mann-Whitney *U* test (**C**) or a Kruskal-Wallis ANOVA (**D**). **Abbreviations**: α-Syn, α-synuclein; α-Syn pS129, α-synuclein phosphorylated Serine 129; CTRL, Control; EEV, erythrocyte-derived extracellular vesicle; H&Y, Hoehn and Yahr; PD, Parkinson's disease.

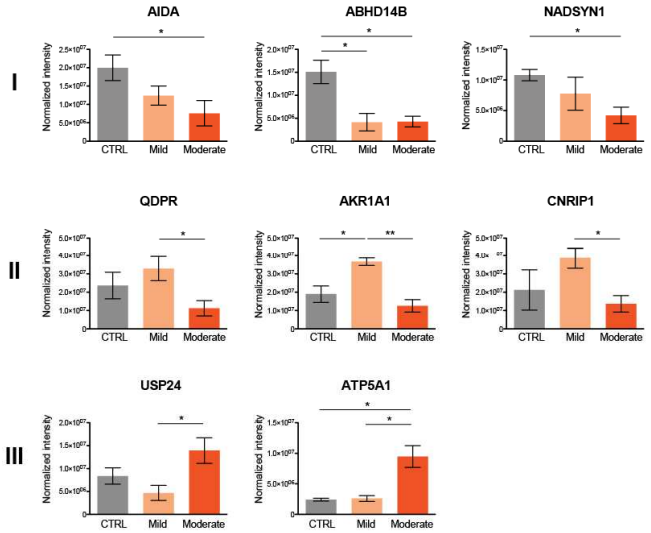
A



Total of 818 proteins identified
8 proteins significantly modified

Groups	Proteins	Genes
I	Axin interactor, dorsalization-associated protein	AIDA
	Alpha/beta hydrolase domain-containing protein 14B	ABHD14B
	Glutamine-dependent NAD(+) synthetase	NADSYN1
II	Dihydropteridine reductase	QDPR
	Alcohol dehydrogenase [NADP(+)]	AKR1A1
	CB1 cannabinoid receptor-interacting protein 1	CNRIP1
III	Ubiquitin carboxyl-terminal hydrolase 24	USP24
	ATP synthase subunit alpha, mitochondrial	ATP5A1

B



C

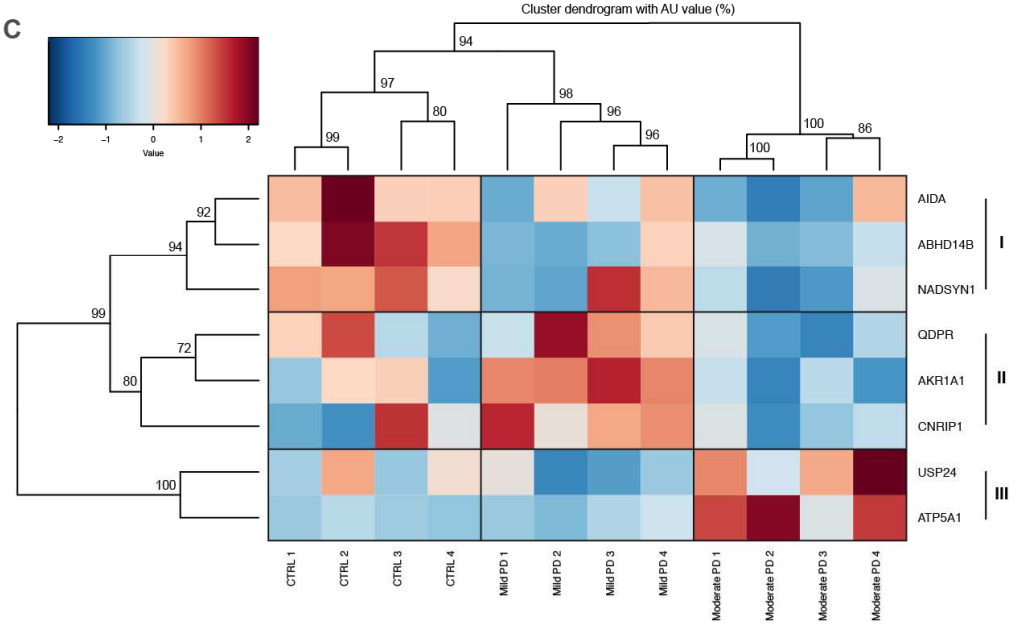


Figure 2.3. Specific protein signature of EEV in PD patients. **A**. NanoLC-MS/MS Label-free analysis of EEV in PD patients and healthy age-matched CTRL revealed a total of 818 proteins, of which 8 had an expression that was significantly modified as a function of PD state (**B**). These proteins have been categorized and referenced according to the gene to which they are associated (**A** – see table). **B**. The proteins/genes have been further separated into 3 groups in relation to their variations in comparison to CTRL (Group I), mild PD (Group II) or moderate PD (Group III). **C**. Heatmap establishing correlations between disease stages and the abundance of the variable proteins. Cold and hot colours represent low and high correlation levels, respectively. The AU *p* value is indicated for each node. Protein level changes were determined by Welch's test p value < 0.05 and absolute value of z-score > 1.96, $*p<0.05$, $**p<0.01$. **Abbreviations**: AU, Approximately Unbiased; CTRL Control; PD, Parkinson's disease.

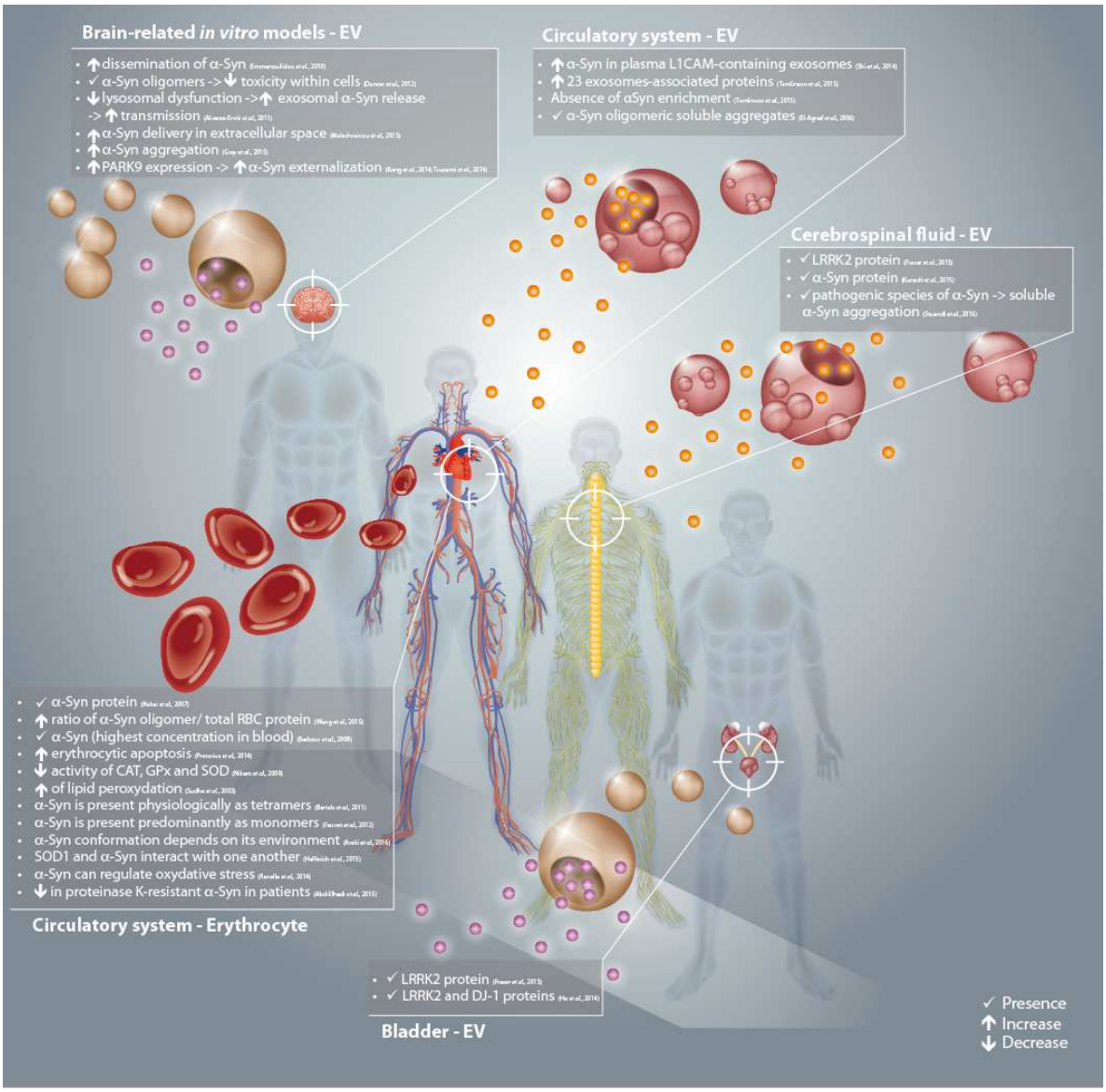


Figure 2.4. Erythrocyte and EV implication in PD: **summary of the literature.** The search for a reliable biomarker in PD has included investigations in neuronal cells in specific *in vitro* systems, in cellular elements within the circulatory system, the cerebrospinal fluid and urine with a targeted interest on the expression of α-Syn. However, none of these studies have convincingly identified a robust marker of PD. **Abbreviations**: αSyn, α-synuclein; CAT, Catalase; GPx, glutathione peroxidase; L1CAM, L1 cell adhesion molecule; LRRK2, Leucine-rich repeat kinase 2; RBC, red blood cells; SOD, superoxide dismutase; ✓ presence; ↑ increase; ↓ decrease.

2.10 Matériels supplémentaires

2.10.1 Materials and methods

Preparation of platelet-free plasma and EV labeling

For all experiments, diluted annexin-V buffer (BD Pharmingen, Mississauga, ON, Canada) and phosphate buffered saline (PBS) were filtered on 0.22mm pore size membranes. To quantify EV according to their cell of origin, the following surface markers were used: CD235a+ (erythrocytes) (5µl), CD31+/CD41- (endothelial cells) (1µl), CD41+ (platelets) (5µl), CD45+ (leukocytes) (3µl), CD45+CD14+ (monocytes) (10µl), and CD45+CD15+ (granulocytes) (2µl), with or without annexin-V staining (5µl). PFP (5µl) was incubated with Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK) 10mM (Calbiochem, Etobicoke, ON, Canada) for 5 minutes followed by a 30-minute incubation with antibodies and annexin-V in a final PBS volume of 100µl, all at room temperature. Finally, samples were diluted to a final volume of 2ml prior to FACS analysis. The following antibodies were purchased from BD Pharmingen and used for all analyses: FITC-conjugated mouse anti-human CD235a (clone GA-R2 (HIR2), 1/20), PE-conjugated mouse anti-human CD31 (clone WM59, 1/100), V450-conjugated mouse anti-human CD41a (clone HIP8, 1/20), V450-conjugated mouse anti-human CD45 (clone HI30, 1/33), APC mouse anti-human CD14 (clone M5E2, 1/10), PE-conjugated mouse anti-human CD15 (clone HI98, 1/50), V450- and PerCP-Cy^{TM}5.5-conjugated annexin-V (1/20).

Flow cytometry quantification

Between sample analyses of the PD and HD patients, the blue laser had to be replaced for maintenance issues and therefore laser settings were recalibrated. For FSC-PMT, the assigned voltage was 363 (PD) and 160 (HD) Volts. For SSC, the assigned voltage was 407 (PD) and 300 (HD) Volts. All other parameters were set between 450 and 500 Volts. The acquisition of EV was performed at low speed at an approximated rate of 10µl/min. To determine background noise levels, antibody mixes were incubated in the absence of PFP sample and unlabeled PFP was used as a negative control. Every sample was re-identified to ensure that the experimenter was blind to clinical status.

Production and purification of EEV

Briefly, the erythrocyte pellet was activated with 3 volumes of calcium ionophore solution (150mM NaCl; 10mM Tris-HCl; 1mM CaCl2; 5µM ionophore A23187 (Sigma, St Louis, MO)) for 30 minutes at 37°C. The activation was stopped by the addition of 5mM EDTA. Remaining erythrocytes were pelleted at 15 000g for 20 minutes. The EEV were centrifuged at 20 000g for 90 minutes and washed once in PBS. The EV pellet was re-suspended in PBS and frozen at -80°C until further analyses.

Scanning electron microscopy

Preparations of erythrocytes (5µl) were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in PBS buffer at least 24 hours before standard dehydration. Samples were washed 3 times for 10 minutes with sodium cacodylate buffer (0.1M, pH 7.3) and fixed with 1% osmium tetroxide in sodium cacodylate buffer for 90 minutes. Subsequently, samples were washed and processed in 50%, 70%, 90% and 100% EtOH for dehydration (10 minutes/step). Finally, samples were soaked in two subsequent baths of 100% EtOH, for 40 minutes and 10 minutes, air-dried overnight and coated with palladium. Observations were completed using a JEOL 6360LV scanning electron microscope (JEOL, Peabody, MA, USA).

Transmission electron microscopy

Preparations of EEV (30µl) and activated erythrocytes (5µl) were fixed in 2% paraformaldehyde at least 24 hours before being dehydrated and sealed in LR white. Slices of LR white were placed on a Formvar/carbon-coated grid and processed for immunolabeling. The tissues mounted on grids were blocked in 0.5% BSA-c (Aurion, Wageningen, The Netherlands) in HBSS and incubated for 120 minutes with rabbit anti-α-Syn antibody (Abcam, Toronto, ON, Canada) or rabbit anti-α-Syn (phospho S129) antibody (Abcam, Toronto, ON, Canada), both diluted at 1:250 in HBSS and washed several times with distilled water. Finally, the grids were incubated for 60 minutes with an anti-rabbit IgG conjugated to 6nm gold particles (EMS, Hatfield, PA, USA) diluted at 1:200, washed several times with distilled water and then fixed in 2.5% glutaraldehyde (EMS, Hatfield, PA, USA) in HBSS for 15 minutes. For this last step, the grids were treated with 3% uranyl acetate–

0.075 M oxalate (pH 7.0) (EMS, Hatfield, PA, USA) for 1 minute, which was followed by several washes in distilled water. All staining experiments included negative controls where the primary antibody was omitted from the incubation media. Observations were completed with a TECNAI Spirit G2 transmission electron microscope at 80kV (FEI, Hillsboro, OR, USA).

Mass spectrometry analysis and label free protein quantification

The extracted peptides from the 7 slices of the same individual were pooled and analyzed by nanoLC-MS/MS. The excised hemoglobin gel slices were also analyzed in the same conditions. One µg of each individual sample was injected on a Dionex UltiMate 3000 nanoRSLC system (Thermo Scientific) equipped with a nanoviper Acclaim Pepmap100, C18, 3µm, 75µm x 50cm column (Thermo Scientific) connected to the nanoelectrospray source of an Orbitrap Fusion mass spectrometer (Thermo Scientific). The peptides were eluted at 300nL/min using an acetonitrile gradient of 90 minutes with the mass spectrometer operating in the Data Dependent Acquisition mode. Peptide masses were measured in MS spectra detected in the orbitrap at 120K resolution. MSMS fragmentation spectra of peptides were generated by Higher energy Collisional Dissociation (HCD) and detected in the ion trap.

Statistical analyses

Initial statistical analyses (unpaired t-tests or Mann-Witney when data followed normal distribution and Shapiro-Wilk test in cases of non-normality) compared the number of each type of blood cells (erythrocytes, platelets, leukocytes, neutrophils, monocytes, lymphocytes) in control groups and patients of all stages of the UK to the Canadian cohorts. This revealed no statistical differences between groups, allowing us to pool cohorts for subsequent analyses. Statistical analyses pertaining EEV quantification were performed using *The Statistics and Machine Learning Toolbox* provided by MathWorks™ under MATLAB™ platform using the MATLAB®R2015a version. Results obtained include the scatter plots, classical least-square linear regressions, R-squared and *p* values, as well as Pearson's goodness-of-fit. Interval cut-off values were determined using a loop program developed in MATLAB™. Model diagnostics, including residual behaviour and homoscedastivity, were

obtained using the same Toolbox. Further details on the statistical tests chosen are described directly in the result section. For α-Syn quantification, data were first tested for normality using the D'Agostino & Pearson normality test. Comparisons between groups were obtained by Mann-Whitney *U* test or Kruskal-Wallis ANOVA and performed using Prism 6.0 (GraphPad Software, LaJolla, CA). For proteomic analyses, the 'Intensity values' contained in the output 'proteingroup.txt' file of MaxQuant were used to quantify each identified protein in each individual sample. The values were normalized by the median of each column (all intensity values of proteins for one sample). The missing values were imputed with a noise value corresponding to the 1-percentile of each sample column. For each comparison between two groups (Control, mild PD or moderate PD), proteins with too many imputed values where considered not quantifiable (a minimum of three not-imputed values in one of the 2 groups are required). A protein ratio was calculated between the two groups using the average of intensity values in each group. Finally, a statistical Welch's test was performed between the two groups. The protein ratios were transformed into log2(ratio) then centered by calculation of a z-score (z-score = $(x - \mu)/\sigma$). A protein was considered as variant if it fulfilled the following criteria: minimum of 2 peptides quantified, Welch's test p value < 0.05 and absolute value of z-score > 1.96 (corresponding to values outside of the 95% confidence interval). The Gene Ontology enrichment analysis on the identified proteins was performed on the Cytoscape platform (v. 3.4.0) using the BinGO software version 3.0.3 against all human genes with GO annotation (Uniprot-GOA generated 2015-06-22) (Maere et al., 2005). Enrichment was calculated by a hypergeometric test and Bonferroni Family-Wise Error Rate (FWER) was used to correct for multiple testing. The data for the resulting 8 proteins was standardized, hierarchically clustered and visualized as a heatmap by using the statistical framework R (R Core Team, 2016). The robustness of the nodes was evaluated by computing Approximately Unbiased (AU) p values using the R package pvclust (10000 bootstraps, average method and correlation-based dissimilarity matrix) (Suzuki and Shimodaira, 2006).

2.10.2 Tableaux

Table S2.1. Quantification of cell-derived EV - PD cohort

Cell types	Markers	Units	CTRL			PD			*p* value
			n	Mean	SEM	*n*	Mean	SEM	
Erythrocytes	**CD235a+PS-**	**X 10^3/µl**	**36**	**18.2**	**46.5**	**59**	**32.0**	**36.3**	**0.04**
	CD235a+PS+	X 10^3/µl	36	0.22	0.07	59	0.29	0.05	0.70
	CD235a+ total	**X 10^3/µl**	**36**	**18.4**	**47.0**	**59**	**32.3**	**36.7**	**0.04**
	EV CD235a+/erythrocyte		**34**	**0.0039**	**0.011**	**57**	**0.0069**	**0.008**	**0.04**
Platelets	CD41+PS-	X 10^3/µl	37	7.88	1.68	59	10.3	1.33	0.27
	CD41+PS+	X 10^3/µl	37	15.2	3.20	59	17.9	2.53	0.51
	CD41+ total	X 10^3/µl	37	23.1	4.62	59	28.2	3.66	0.38
	EV CD41+/platelet		35	0.106	0.021	57	0.125	0.016	0.49
Endothelial cells	CD31+CD41-PS-	X 10^3/µl	37	15.8	8.04	59	11.7	6.37	0.75
	CD31+CD41-PS+	X 10^3/µl	37	0.91	0.13	59	0.92	0.10	0.96
	CD31+CD41- total	X 10^3/µl	37	16.7	8.03	59	12.6	6.36	0.75
Leukocytes	CD45+ total	X 10^3/µl	37	10.4	2.21	59	13.8	1.75	0.26
Monocytes	CD45-CD14+PS-	X 10^3/µl	37	1.70	0.30	59	1.62	0.24	0.85
	CD45-CD14+PS+	X 10^3/µl	37	1.20	4.00	59	5.84	3.17	0.50
	CD45+CD14+PS-	X 10^3/µl	37	0.16	0.04	59	0.14	0.03	0.74
	CD45+CD14+PS+	X 10^3/µl	37	0.60	0.79	59	1.47	0.63	0.59
	CD14+ total	X 10^3/µl	37	3.66	4.88	59	9.06	3.87	0.60
	EV CD14+/monocyte		35	7.08	1.99	57	9.16	1.56	0.41
Granulocytes	CD45-CD15+PS-	X 10^3/µl	37	12.3	7.96	59	16.7	6.30	0.92
	CD45-CD15+PS+	X 10^3/µl	37	2.21	0.77	59	1.39	0.61	0.47
	CD45+CD15+PS-	X 10^3/µl	37	0.55	0.36	59	1.15	0.29	0.20
	CD45+CD15+PS+	X 10^3/µl	37	1.01	0.30	59	1.25	0.24	0.56
	CD15+ total	X 10^3/µl	37	16.0	8.83	59	20.6	6.99	0.91
	EV CD15+/granulocyte		35	3.70	0.64	57	3.16	0.50	0.53

Table S2.1. Quantification of cell-derived EV – PD cohort. The quantification of EV in platelet-free plasma of PD patients and controls was performed by high-sensitivity flow cytometry. Phosphatidylserine was evaluated with Annexin V binding. The complete blood count was obtained at the time of blood sampling for 35|37 Controls and 57|59 PD and was used to calculate the EV on cell ratio. * For erythrocyte-derived EV quantification, 1 outlier (determined using Grubbs' method with alpha=0.0001) was removed. As opposed to **Figure 3A**, all other samples were included in the analyses (i.e. elevated hemoglobin, diabetes and/or patients having suffered from cancer). **Abbreviation:** CD235a, glycophorin A; CTRL, Control; EV, extracellular vesicle; PD, Parkinson's disease; PS, phosphatidylserine.

Table S2.2. Quantification of cell-derived EV - HD cohort

Cell types	Markers	Units	CTRL			HD pre-manifest			HD			*p* value
			n	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	
Erythrocytes	CD235a+PS-	X 10^3/µl	54	15.2	2.0	10	10.3	3.5	51	14.1	1.4	0.16
	CD235a+PS+	**X 10^3/µl**	54	1.1	0.2	**10**	**0.4**	**0.2**	**51**	**1.1**	**0.1**	**0.04**
	CD235a+ total	X 10^3/µl	54	16.4	2.0	10	10.7	3.5	51	15.3	1.5	0.09
	EV CD235a+/erythrocyte		54	0.0035	0.0005	10	0.0023	0.0008	50	0.0033	0.0003	0.11
Platelets	CD41+PS-	X 10^3/µl	54	9.2	2.2	10	4.3	1.3	50	6.1	1.0	0.78
	CD41+PS+	X 10^3/µl	54	19.3	4.8	10	7.1	2.0	50	12.4	2.4	0.74
	CD41+ total	X 10^3/µl	54	28.4	6.9	10	11.4	3.2	50	18.6	3.4	0.70
	EV CD41+/platelet		53	0.12	0.03	10	0.05	0.02	48	0.08	0.01	0.34
Endothelial cells	CD31+CD41-PS-	X 10^3/µl	54	1.4	0.3	10	0.6	0.2	50	1.2	0.2	0.31
	CD31+CD41-PS+	X 10^3/µl	54	0.68	0.16	10	0.25	0.06	50	0.46	0.09	0.59
	CD31+CD41- total	X 10^3/µl	54	2.1	0.4	10	0.8	0.2	50	1.7	0.3	0.26
Leukocytes	CD45+ total	X 10^3/µl	54	33.4	2.7	10	31.6	5.3	51	31.7	2.4	0.88
Monocytes	CD45-CD14+PS-	X 10^3/µl	54	3.4	1.1	10	1.6	0.2	51	1.6	0.1	0.91
	CD45-CD14+PS+	X 10^3/µl	54	1.8	0.3	10	0.8	0.3	51	1.5	0.2	0.14
	CD45+CD14+PS-	X 10^3/µl	54	0.18	0.07	10	0.069	0.016	51	0.056	0.008	0.34
	CD45+CD14+PS+	X 10^3/µl	54	0.62	0.12	10	0.24	0.06	51	0.55	0.14	0.12
	CD14+ total	X 10^3/µl	54	6.0	1.3	10	2.6	0.4	51	3.7	0.4	0.08
	EV CD14+/monocyte		53	12.3	2.5	10	5.7	0.6	48	8.0	1.0	0.13
Granulocytes	CD45-CD15+PS-	X 10^3/µl	54	1.2	0.1	10	1.2	0.3	51	1.5	0.2	0.33
	CD45-CD15+PS+	X 10^3/µl	54	0.12	0.04	10	0.18	0.08	51	0.22	0.11	0.33
	CD45+CD15+PS-	X 10^3/µl	54	0.20	0.05	10	0.07	0.02	51	0.15	0.04	0.64
	CD45+CD15+PS+	X 10^3/µl	54	0.25	0.05	10	0.13	0.06	51	0.20	0.04	0.39
	CD15+ total	X 10^3/µl	54	1.7	0.2	10	1.6	0.4	51	0.20	0.3	0.67
	EV CD15+/granulocyte		53	0.41	0.04	10	0.42	0.13	48	0.50	0.08	0.75

Table S2.2. Quantification of cell-derived EV – HD cohort. The quantification of EV in platelet-free plasma of HD patients and controls was performed by high-sensitivity flow cytometry. Phosphatidylserine was evaluated with Annexin V binding. The complete blood count was obtained at the time of blood sampling for 53|54 CTRL, 10 pre-manifest and 48|50|51 HD patients and was used to calculate the EV on cell ratio. Statistical analyses were performed using Wilcoxon/Kruskal-Wallis followed by Wilcoxon's multiple comparison tests. **Abbreviation**: CD235a, glycophorin A; CTRL, Control; EV, extracellular vesicle; HD, Huntington's disease; PS, phosphatidylserine.

Voir Annexe

Table S2.3. Complete list of proteins identified in the EEV proteome. Lists of the protein groups identified by LC-MS/MS analysis using Andromeda/MaxQuant search engine in the Uniprot Complete Proteome *Homo sapiens* database for the non-depleted sample (356 proteins) (**A**) or when hemoglobin was analyzed separately (818 proteins) (**B**). The lists were filtered at 1% False Discovery Rate using a target/decoy database search.

2.10.3 Figures

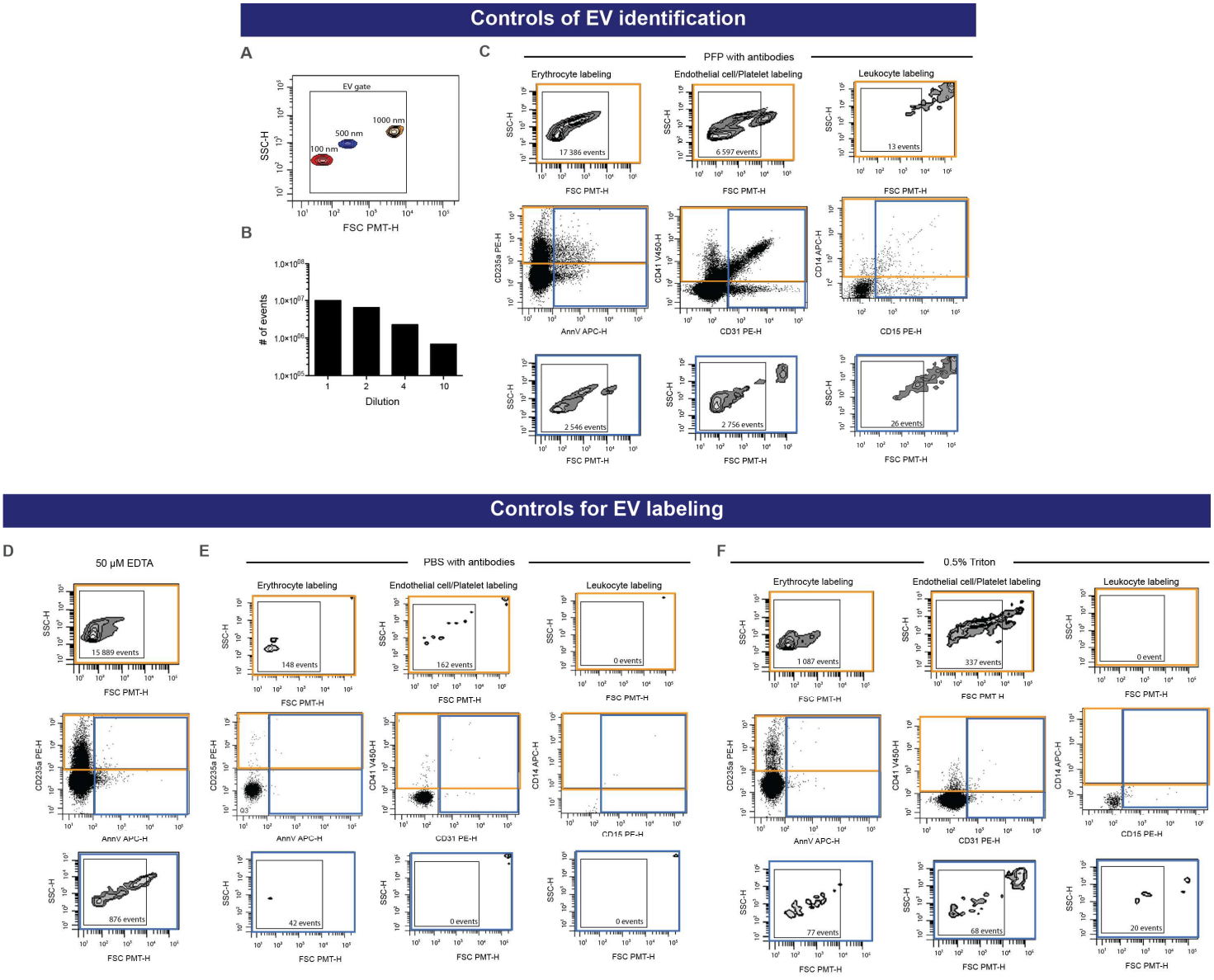
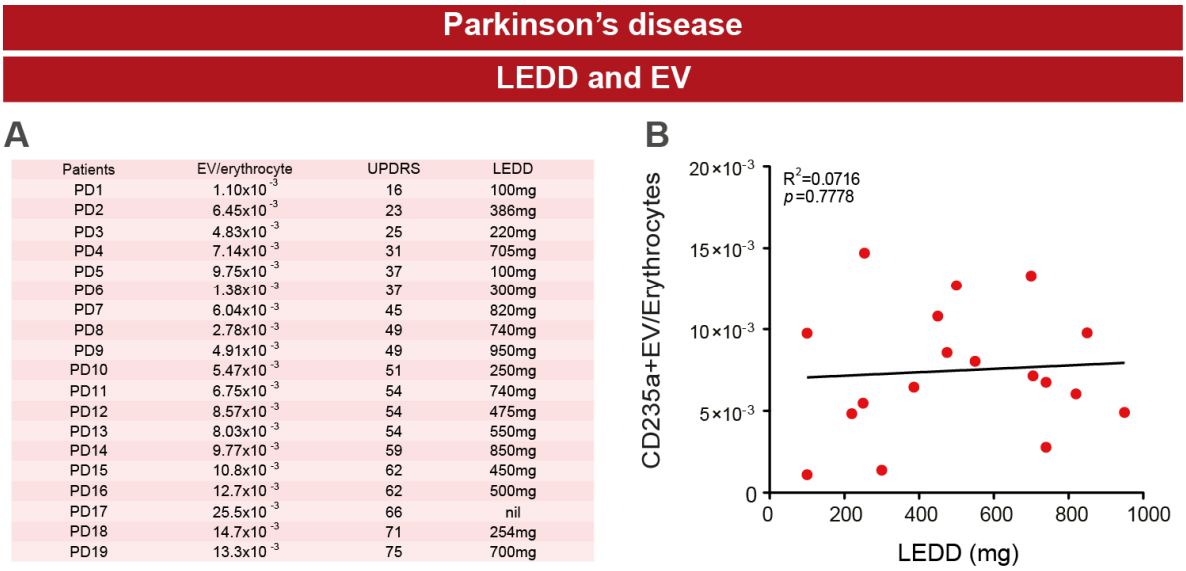


Figure S2.1. Optimization of EV detection: controls for flow cytometry. *Controls for EV identification.* **A**. To properly set the EV gate, fluorescent silica beads of 100nm (*Red*), 500nm (*Blue*) and 1000nm (*Yellow*) were acquired on a flow cytometer Canto II modified with a FSC-PMT small particle option. The established EV gate was used for all experiments. **B**. Serial dilutions of EEV (1, 2, 4 and 10) were used to confirm the linearity of the quantification. **C**. FSC-PMT/SSC gates of PFP stained with annexin V and respective fluorochrome-conjugated antibodies directed against erythrocyte (CD235a+), endothelial (CD31+/CD41-), platelet (CD41+) and leukocyte (CD14+CD45+, monocytes;

CD15+CD45+, granulocytes)-derived EVs. *Controls for EV labeling*. **D**. Treatment with the ion chelator EDTA inhibited the binding of annexin V to phosphatidylserine. **E**. Minimal background was observed using antibodies in the absence of PFP. This background was subtracted from all subsequent EV quantifications. **F**. EV sensitivity to 0.5% triton was further assessed. **Abbreviations**: AnnV, annexinV; EV, extracellular vesicle; EEV, erythrocyte-derived extracellular vesicle; FSC PMT-H, forward scatter photomultiplier tube; PBS, phosphate buffered saline; PFP, platelet free plasma; SSC-H, side scatter.



Patients	EV/erythrocyte	UPDRS	LEDD
PD1	$1.10x10^{-3}$	16	100mg
PD2	$6.45x10^{-3}$	23	386mg
PD3	$4.83x10^{-3}$	25	220mg
PD4	$7.14x10^{-3}$	31	705mg
PD5	$9.75x10^{-3}$	37	100mg
PD6	$1.38x10^{-3}$	37	300mg
PD7	$6.04x10^{-3}$	45	820mg
PD8	$2.78x10^{-3}$	49	740mg
PD9	$4.91x10^{-3}$	49	950mg
PD10	$5.47x10^{-3}$	51	250mg
PD11	$6.75x10^{-3}$	54	740mg
PD12	$8.57x10^{-3}$	54	475mg
PD13	$8.03x10^{-3}$	54	550mg
PD14	$9.77x10^{-3}$	59	850mg
PD15	$10.8x10^{-3}$	62	450mg
PD16	$12.7x10^{-3}$	62	500mg
PD17	$25.5x10^{-3}$	66	nil
PD18	$14.7x10^{-3}$	71	254mg
PD19	$13.3x10^{-3}$	75	700mg

Figure S2.2. LEDD and EEV. Additional information on patients derived from each correlation has been provided (number of EEV/total number of erythrocytes and LEDD) to illustrate that LEDD cannot account for the differences we found in EEV counts. **A**. EV/erythrocytes ratios, UPDRS scores and LEDD values for all PD patients used in correlation analyses. **B**. Absence of correlation between the number of EEV and LEDD. **Abbreviations**: EEV, erythrocyte-derived extracellular vesicle; EV, extracellular vesicle; LEDD, Levodopa equivalent daily dose; UPDRS, Unified Parkinson's Disease Rating Scale.

A

Blood cell quantification – PD

Cell type	Units	CTRL			Unknown			Mild			Moderate			Severe			*p* value
		n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	
Erythrocytes	10^9/ml	35	4.75	0.61	6	4.63	0.47	12	4.44	0.59	31	4.62	0.44	8	4.54	0.38	0.50
Platelets	10^6/ml	35	235	44	6	217	48	12	221	40	31	220	51	8	205	63	0.51
Leukocytes	10^6/ml	35	4.89	1.44	6	4.65	1.46	12	4.08	1.49	31	4.8	1.25	8	6.65	2.69	0.23
Monocytes	10^6/ml	35	0.56	0.14	6	0.48	0.2	12	0.54	0.19	31	0.51	0.17	8	0.59	0.19	0.64
Lymphocytes	10^6/ml	35	1.78	0.54	6	1.65	0.56	12	1.57	0.73	31	1.48	0.4	8	1.37	0.52	0.14
Neutrophils	10^6/ml	35	4.1	1.34	6	4.0	1.37	12	3.34	1.27	31	4.12	1.1	8	5.83	2.71	0.18

B

Blood cell quantification – HD

Cell type	Units	CTRL			Pre-HD			Stage 1			Stage 2			Stage 3			Stage 4			Stage 5			*p* value
		n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	n	Mean	SD	
Erythrocytes	10^9/ml	54	4.58	0.36	10	4.59	0.36	15	4.75	0.37	12	4.56	0.29	11	4.69	0.44	10	4.76	0.39	2	4.35	0.26	0.44
Platelets	10^6/ml	54	229	51	10	259	85	15	232	68	12	218	54	11	268	63	10	212	46	2	305	55	0.09
Leukocytes	10^6/ml	54	6.61	1.60	10	6.97	1.64	15	6.3	1.46	12	7.67	2.97	11	6.97	2.0	10	6.84	2.07	2	5.3	1.56	0.47
Monocytes	10^6/ml	54	0.51	0.16	10	0.48	0.17	15	0.49	0.13	12	0.55	0.18	10	0.58	0.24	10	0.51	0.16	2	0.61	0.42	0.79
Lymphocytes	10^6/ml	54	1.83	0.52	10	2.1	0.57	15	1.67	0.5	12	1.79	0.62	10	1.78	0.52	10	1.81	0.79	2	1.45	0.78	0.62
Neutrophils	10^6/ml	54	4.06	1.29	10	4.19	1.17	15	3.96	1.29	12	5.13	2.49	10	4.51	1.67	10	4.33	1.41	2	3.03	1.16	0.31

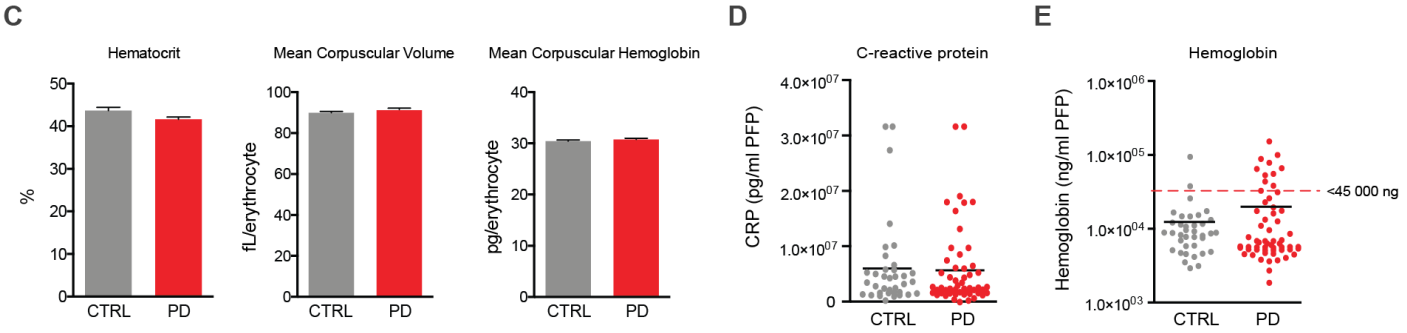


Figure S2.3. Blood counts and exclusion criteria. **A-B**. Full blood counts performed in PD (*n*=57), pre-manifest HD (*n*=10) and HD patients (*n*=50) revealed no significant differences for the total number of any cell type between patients and their respective healthy sex- and age-matched CTRL (*n*=37 for PD; *n*=55 for pre-HD/HD). **C**. Evaluated hematocrit, mean corpuscular hemoglobin and mean corpuscular volumes were similar between groups (PD, *n*=43; CTRL, *n*=24). **D-E**. CRP (indicative of an inflammatory response) (**D**) and free hemoglobin (indicative of hemolysis) (**E**) were further quantified in the PFP (PD, *n*=59; CTRL, *n*=37). Individuals presenting with levels exceeding 45 000ng/ml were excluded from the study to remove the possibility that these factors contributed to increased EEV concentrations. Statistical analyses were performed using Kruskal-Wallis ANOVAs (**A-B**) and Mann-Whitney *U* tests (**C-E**). **Abbreviations**: CTRL, controls; CRP, C-reactive protein; EEV, erythrocyte-derived extracellular vesicle; HD, Huntington's disease; PD, Parkinson's disease; PFP, platelet-free plasma.

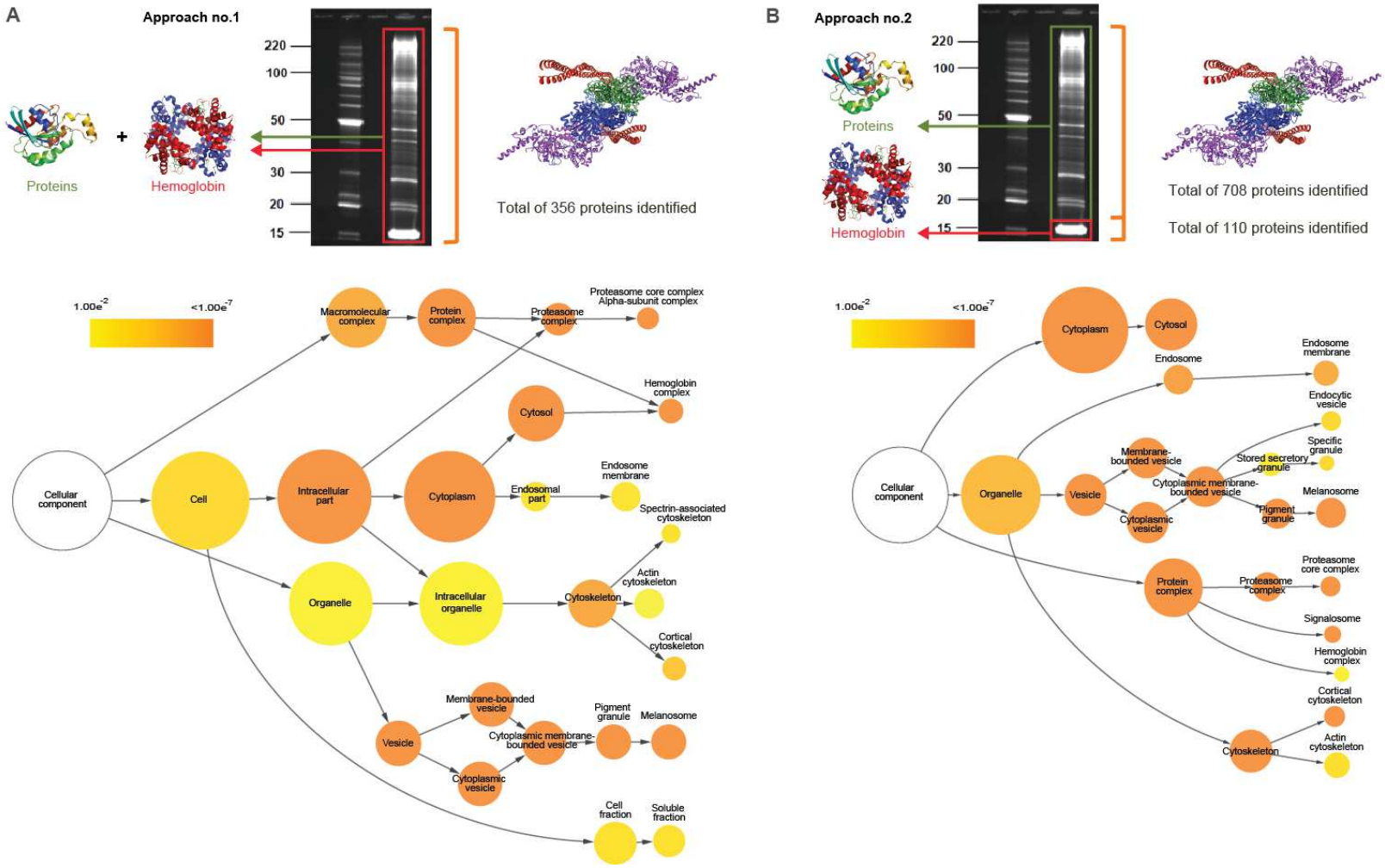

A
Approach no.1
Proteins
Hemoglobin
220
100
50
30
20
15
Total of 356 proteins identified
1.00e-2
<1.00e-7
Cellular component
Cell
Organelle
Macromolecular complex
Protein complex
Proteasome complex
Proteasome core complex
Alpha-subunit complex
Hemoglobin complex
Intracellular part
Cytoplasm
Cytosol
Endosomal part
Endosome membrane
Spectrin-associated cytoskeleton
Intracellular organelle
Cytoskeleton
Actin cytoskeleton
Cortical cytoskeleton
Vesicle
Membrane-bounded vesicle
Cytoplasmic membrane-bounded vesicle
Pigment granule
Melanosome
Cytoplasmic vesicle
Cell fraction
Soluble fraction
B
Approach no.2
Proteins
Hemoglobin
220
100
50
30
20
15
Total of 708 proteins identified
Total of 110 proteins identified
1.00e-2
<1.00e-7
Cellular component
Organelle
Cytoplasm
Cytosol
Endosome
Endosome membrane
Endocytic vesicle
Specific granule
Stored secretory granule
Membrane-bounded vesicle
Cytoplasmic membrane-bounded vesicle
Vesicle
Cytoplasmic vesicle
Pigment granule
Melanosome
Protein complex
Proteasome complex
Proteasome core complex
Signalosome
Hemoglobin complex
Cytoskeleton
Cortical cytoskeleton
Actin cytoskeleton

Figure S2.4. Proteomic analyses with and without hemoglobin. To ensure the specificity of the protein signature detected in EEV, we performed proteomic analyses using two distinct methodologies. **A**. The first set of analyses by nanoLC-MS/MS and Label Free Quantification was performed on the complete proteome of the EEV, yielding 356 proteins. **B**. The second set of analyses was also performed on the entire EEV proteome but this time, isolating hemoglobin and quantifying the proteins present in both fractions. This approach uncovered 708 proteins in the EEV proteome with an additional 110 proteins in the hemoglobin fraction for a total of 818 proteins, indicating that removing hemoglobin provides a much more accurate evaluation of the protein content of EEV. To further confirm that the identified proteins were associated with the EEV proteome, a Gene Ontology enrichment analysis was performed on the Cellular Component ontology using BinGO software. The list of 356 identified proteins in the entire EEV (**A**) and the list of the 818 identified proteins when hemoglobin was analyzed separately (**B**) were compared to the whole human proteome (reference list). Circle size is proportional to the number of proteins matching the corresponding GO term and the color scale corresponds to the *p* value of enrichment (darker orange corresponds to greater statistical enrichments).

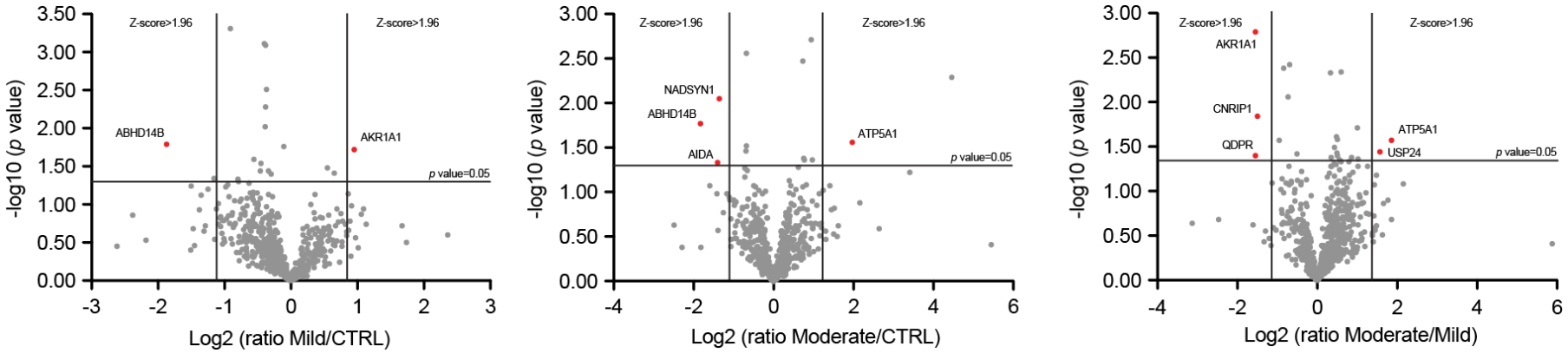


Figure S2.5. Confirmation of EEV proteins selectively modified in PD patients by Volcano plots. The protein ratios (log2(ratio)) of the three comparisons (mild PD/CTRL, moderate PD/CTRL and moderate PD/mild PD) were plotted over the corresponding Welch's test *p* value (-log10(p-value)). The graphs display a V shape, as expected, and only the proteins falling outside the limits of a *p* value < 0.05 and absolute value of z-score > 1.96 (identified by black lines) were considered as variant proteins (red dots). Two variant proteins were excluded given that they were quantified using only one peptide. **Abbreviations**: CTRL: Control; PD, Parkinson's disease.